PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 35/14, 37/04, C07K 15/22 C07K 13/00, C12P 21/02 C12N 5/00

A1

(11) International Publication Number: WO 91/14438

(43) International Publication Date: 3 October 1991 (03.10.91)

(21) International Application Number: PCT/US91/01844

(22) International Filing Date: 20 March 1991 (20.03.91)

(30) Priority data:
496,409 20 March 1990 (20.03.90) US

(60) Parent Application or Grant
(63) Related by Continuation
US 496,409 (CIP)
Filed on 20 March 1990 (20.03.90)

(71) Applicant *(for all designated States except US):* THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK [US/US]; Broadway and West 116th Street, New York, NY 10027 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : MORRISON, Sherie, L. [US/US]; 258 Denslow Avenue, Los Angeles, CA 90048 (US). SHIN, Seung-Uon [US/US]; 7700 Topanga Canyon, Canoga Park, CA 91304 (US).

(74) Agent: WHITE, John, P.; Cooper & Dunham, 30 Rockefeller Plaza, New York, NY 10112 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: CHIMERIC ANTIBODIES WITH RECEPTOR BINDING LIGANDS IN PLACE OF THEIR CONSTANT REGION

(57) Abstract

The present invention provides a modified chimeric monoclonal antibody comprising two molecules of each of two different polypeptides. The shorter polypeptides function as the light chains of the antibody and the longer polypeptides function as the heavy chains of the antibody. Moreover, the polypeptide which functions as a heavy chain has a variable region characteristic of a first mammal and a constant region characteristic of a second mammal. Each polypeptide which functions as a light chain has a variable region characteristic of a mammal and a constant region characteristic of a mammal, wherein a receptor-binding ligand replaces at least a portion of the constant region of each of the polypeptides which function as the heavy chains of the antibody. Additionally, the present invention provides an immunologically reactive complex and a chimeric polypeptide. Finally, methods of using and producing the modified chimeric monoclonal antibodies, immunologically reactive complexes, and chimeric polypeptides are provided herein.

ATTORNEY DOCKET NUMBER: 10271-027-999
SERIAL NUMBER: 10/020,354
REFERENCE: AM

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

CHIMERIC ANTIBODIES WITH RECEPTOR BINDING LIGANDS IN PLACE OF THEIR CONSTANT REGION

This application is a continuation-in-part of U.S. Serial No. 496,409, filed March 20, 1990, the contents of which are hereby incorporated by reference into the subject application.

This invention was made with support under Grant Number NIH-CA16858 from the National Institute of Health, U.S. Department of Health and Human Resources. Accordingly, the U.S. Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

The challenge in cancer therapy has been to find a means of selectively killing malignant cells while leaving normal cells intact. Traditional chemotherapy has been directed against actively dividing cells with a drawback being that it also kills normal, actively proliferating cells, e.g. the bone marrow. Another challenge is to produce antibodies which have access to regions of the subject generally inaccessible to most molecules, e.g., the brain.

Antibodies, because of their remarkable specificity, have long had appeal as the "magic bullet" capable of selectively identifying and eliminating malignant cells.

Transfected cells (transfectomas) provide an approach to improving monoclonal antibodies. Genetically engineered antibodies can be expressed following gene transfection into lymphoid cells (1-5). One of the major advantages of expressing genetically engineered antibodies is that one is not limited to using antibodies as they occur in nature. In particular, nonimmunoglobulin sequences can be joined to antibody sequences, creating multifunctional molecules.

Among the problems encountered when investigators have attempted to use monoclonal antibodies as immunotherapeutic agents is efficiently targeting the antibodies to tumor cells while leaving normal cells untouched. In trying to overcome this problem, we have found that both the growth factor and an anti-tumor specificity can be contained in a single molecule and function synergistically. Therefore, we have joined growth factors to an antibody combining specificity in order to produce molecules which effectively target tumor cells possessing growth factor receptors. Growth factor receptors have also been reported to be on the blood-brain barrier therefore the molecules described herein may be able to utilize growth factor receptors for transcytosis into the brain (18, 49, 15). Growth factors are appropriate because cancer cells usually express growth factor receptors thus reflecting their increased capacity for proliferation.

Presently, antibodies directed to the IL-2 receptor have been used to target therapy to T cell malignancies (67, 68). Alternatively, receptor ligands have been used as

a therapeutic to target a particular cell. Further, blocking cellular receptors effectively interferes with the cells' ability to proliferate.

The molecules described herein include genetically engineered antibodies having insulin or insulin-like growth factors type 1 and type 2 (IGF1 and IGF2) joined to an antibody combining specificity. Insulin and IGF1 and IGF2 are related polypeptides which affect cell metabolism and proliferation by binding to specific receptors on the plasma membrane. IGF1 is identical to somatomedin-C. The receptors for insulin and IGF1 are similar in molecular size and substructure.

Insulin can bind to the IGF1 receptor but binding is weaker than that of IGF1. Recently IGF1 has been shown to be an autocrine growth factor for certain human mammary carcinoma cells in culture (35). Both the insulin and IGF receptors are thought to be present on the blood-brain barrier and to effect transcytosis across it (1, 16-19, 48).

Additionally, the molecules described herein include genetically engineered antibodies having transferrin joined to an antibody combining specificity. Transferrin receptors are widely distributed on human tumors (20). In addition, the vascular endothelium of the brain capillaries express transferrin receptors whereas those of other tissues do not (26). Antibodies directed against transferrin receptors inhibit growth of tumor cells by crosslinking the transferrin receptor (54). However, only IgM antibodies were effective thereby suggesting that a polymer is important. Moreover, IgG antibodies increased the turnover of the transferrin receptor (69) suggesting that an alternative approach to

inhibiting cell growth would be to increase receptor degradation during recycling.

The transferrin receptor binds to the major serum iron transport protein, i.e. transferrin, and mediates iron uptake into the cell. The basic mechanism by which the transferrin receptor mediates iron uptake has been established. After binding of iron-laden transferrin to the receptor, receptor-ligand complexes are taken up via coated pits and accumulate within endocytic vesicles (4, 23, 24). Acidification of this intracellular membrane compartment leads to the dissociation of iron from transferrin and the apotransferrin-transferrin receptor complex is then recycled back to the cell surface. Under neutral conditions, apotransferrin rapidly dissociates from the receptor which is then available to undergo another round of endocytosis (11, 30, 65, 70).

Endocytosis and recycling of receptor occur rapidly and efficiently. Most receptors are returned to the cell surface during each cycle and few are diverted into lysosomes and degraded. The mechanism by which iron is transported into the cytoplasm is unknown. In order to target antibodies to the cytoplasm, antibodies must be resistant to degradation in the endocytic vesicles.

The production of recombinant murine Fab-like antibodies comprising an active enzyme moiety or a polypeptide displaying c-myc antigenic determinants at the constant region of the heavy chain have been reported (43). In contrast, we have joined insulin-like growth factor 1 (IGF1) to a murine anti-dansyl (Dns) combining specificity and the heavy (H) chain constant (C) region C_H2 domain from human IgG3. When this chimeric H chain was transfected into a myeloma cell along with the dansyl-specific light (L) chain, the expected molecule

was produced, assembled, and secreted. The resulting chimeric proteins bound the hapten Dns. They also were bound by the growth factor receptor, but with reduced efficiency, and exhibited some of the functions of IGF1 such as increasing uptake of α-aminoisobutyric acid and 2-deoxy-D-glucose (2-dGlc) (2).

SUMMARY OF THE INVENTION

The present invention provides a modified chimeric monoclonal antibody comprising two molecules of each of two different polypeptides. The shorter polypeptides function as the light chains of the antibody and the longer polypeptides function as the heavy chains of the antibody. Moreover, the polypeptide which functions as a heavy chain has a variable region characteristic of a first mammal and a constant region characteristic of a second mammal. Each polypeptide which functions as a light chain has a variable region characteristic of a mammal and a constant region characteristic of a mammal, wherein a receptor-binding ligand replaces at least a portion of the constant region of each of the polypeptides which function as the heavy chains of the antibody.

Additionally, the present invention provides an immunologically reactive complex comprising two different polypeptides, the shorter of which functions as a light chain and the longer of which functions as a heavy chain. The polypeptide which functions as the heavy chain has a variable region characteristic of a first mammal and a constant region characteristic of a second mammal and the polypeptide which functions as the light chain has a variable region characteristic of a mammal and a constant region characteristic of a second mammal. Moreover, a receptor-binding ligand replaces at least a portion of a constant region of one of the polypeptides.

The invention also provides a chimeric polypeptide capable of functioning as a heavy chain of an antibody. The chimeric polypeptide comprises a variable region characteristic of a first mammal and a constant region characteristic of a second mammal. Moreover, a receptor-

binding ligand replaces at least a portion of the constant region of the polypeptide. Additionally, the present invention provides a chimeric polypeptide capable of functioning as a light chain of an antibody comprising a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand replaces at least a portion of the constant region of the polypeptide.

Moreover, the present invention additionally provides a modified chimeric monoclonal antibody comprising two molecules of each of two different polypeptides, the shorter of which functions as the light chains of the antibody and the longer of which polypeptides function as the heavy chains of the antibody. Each polypeptide which functions as a heavy chain has a variable region characteristic of a first mammal and a constant region characteristic of a second mammal. Further, each polypeptide which functions as a light chain has a variable region characteristic of a mammal and a constant region characteristic of a mammal, wherein a receptor-binding ligand is covalently attached to the ends of the constant regions of each of the polypeptides which function as the heavy chains of the antibody.

Further, another immunologically reactive complex is provided. The complex comprises two different polypeptides, the shorter of which functions as a light chain and the longer of which functions as a heavy chain, the polypeptide which functions as the heavy chain having a variable region characteristic of a first mammal and a constant region characteristic of a second mammal and the polypeptide which functions as the light chain having a variable region characteristic of a mammal and a constant region characteristic of a second mammal, wherein a

receptor-binding ligand is covalently attached to the ends of a constant region of one of the polypeptides.

The present invention also provides a chimeric polypeptide capable of functioning as a heavy chain of an antibody comprising a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand is covalently attached to the constant region of the polypeptide.

Moreover, the present invention further provides a chimeric polypeptide capable of functioning as a light chain of an antibody comprising a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand is covalently attached to the constant region of the polypeptide.

Finally, the invention provides methods of using and producing the modified chimeric monoclonal antibodies.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1.

(A) Strategy for the construction of the IgG3-IGF1 fusion gene. The fourth base (C) of the gene for the C_H2 domain in human IgG3 was mutated to G by site-directed mutagenesis, resulting in the introduction of a unique Pvu II restriction enzyme site. In addition, a unique Pvu II site was introduced into the IGF1 gene by changing the last base (C) of the leader sequence (Leader Seq. or LS) of rat IGF1 cDNA (italic letters) to T. Human IgG3 and rat IGF1 cDNA digested with Pvu II were ligated, resulting in an in-frame IgG2-IGF1 fusion gene without any significant amino acid substitutions.

(B) Schematic representation of the transfection vectors, the genetically engineered IgG3-IGF1 fusion gene and a proposed chimeric antibody. The mouse-human κ L chain gene was cloned into [SV184▲H-neo, which is derived from pACYC184 and contains the pACY origin of replication, a chloramphenicol-resistant gene (Cm^r) for selection in Escherichia coli, and the neo gene (the dotted box) with the simian virus 40 (SV40) early region promoter (the shaded box) for selection in eukaryotic cells. The mouse-human IgG3-rat IGF1 H chain gene is cloned into pSV2▲H-gpt gene (the stippled box). Boxes in the chimeric genes represent exons. The thick black solid line and boxes represent DNA of mouse origin, while the thin solid line and open boxes represent human DNA segments. The shaded box in the H chain genes represents the rat IGF1 cDNA. The sites of cleavage by restriction endonucleases EcoR1 (open triangle), BamHI (open circle), and HindIII (closed circle) are shown. The mouse-human IgG3-rat IGF1 chimeric protein produced by expression of both transfection vectors is shown at the bottom of B. The black region of the chimeric molecule represents the mouse V region domains specific for the hapten Dns, the

open regions represent the C domains of human κ L chain and IgG3, and the hatched region represents the rat IGF1.

Figure 2. NaDodSO$_4$/PAGE analysis of the IgG3-IGF1 chimeric protein secreted by transfectomas. The secreted IgG3-IGF1 chimeric protein biosynthetically labeled with [^{35}S]methionine was analyzed under non reducing **(A)** and reducing **(B)** conditions. The labeled chimeric protein was precipitated with either Dns-Sepharose (lanes DNS in A) or anti-human IgG Fab antiserum/Staphylococcus protein A/IgGsorb (lanes Fab in A). Anti-Fab precipitates free L chains as well as L chains covalently attached to H chains. The secreted IgG3 chimeric antibody consisting of mouse V region-human IgG3 C region has the same basic structure as the IgG3-IGF1 chimeric protein and is used as a control. Under nonreducing conditions (A), the three bands represent the heterogeneous assembly of the processed and unprocessed chimeric protein: under reducing conditions, the processed (P) and unprocessed (unP) chimeric protein are seen (B). The schematic diagrams of heterogeneous assembly patterns are shown in C in which the hatched regions represent the mouse V regions, the open regions represent human C regions, the stippled regions represent the mature IGF1, and the black regions represent the carboxyl terminus of the unprocessed IGF1.

Figure 3. Purification of IgG$_3$-IGF1 fusion protein.
A. Purified IgG$_3$-IGF1 fusion protein was fractionated by SDS-PAGE under non-reducing conditions and visualized using silver staining.
B. The purified IgG$_3$-IGF1 fusion protein was fractionated using FPLC (Superose-12 column, flow rate: 0.25 ml/min). The elution time of IgG$_3$-IGF1 fusion protein is 42 min. The elution time of mouse-human IgG$_3$ chimeric antibody

(170 KDa) is 40 min and that of IgG_1 chimeric antibody (146 KDa) is 45 min; they are indicated by arrows.

Figure 4. Competitive inhibition of binding of ^{125}I-IGF1 to IM-9 lymphocytes. Approximately 3 X 10^6 IM-9 cells were incubated at 15°C with a constant amount of ^{125}I-IGF1 and the indicated concentration of unlabeled competitors (recombinant IGF1, IgG3-IGF1 chimeric protein and IgG3 chimeric antibody). After 2 hr of incubation, the amount of receptor-bound radioactivity was determined. Values are expressed as the relative inhibition of binding compared to use of only labeled tracer ^{125}I-IGF1. Results shown for each curve are the means of duplicate experiments.

Figure 5. Relationships between IGF1 and IgG3-IGF1 stimulatory effects on α-aminoisobutyric acid (AIB) **(A)** and dGlc **(B)** uptakes in KB cells. Uptakes were determined in the presence of the indicated concentrations of IGF1, IgG3-IGF1, and IgG3 as control.

Figure 6. Schematic diagram showing the differential centrifugation protocol which was used to obtain a nuclear/plasma membrane (Nuc/Pm) fraction, a Mitochondria (Mit) fraction, a high density microsomes (H. Micro) fraction, and a low density microsomes (L. Micro) and cytosolic (Cyto) fraction.

Figure 7. Diagram of pAG5018 comprising hinge-IL2 in an expression vector.

Figure 8. Line graph showing the relative activity of TUZ (an IL-2 fusion protein) versus IL-2.

Figure 9. Schematic of the Bluescript (KS) with the CD4-IGF1 insert.

Figure 10. Schematic of pSV2 V_{DNS} HUG3 HTF comprising human transferrin.

Figure 11. Line graph showing IgG3-IGF1 Capillary Depletion.

DETAILED DESCRIPTION OF THE INVENTION

Many problems have been encountered in the attempt to use monoclonal antibodies as immunotherapeutic agents. Among the problems have been the inability to (1) efficiently target antibodies to tumor cells while leaving normal cells untouched and (2) gain access to restricted locations in the body, e.g. the brain.

Accordingly, the monoclonal antibodies provided herein are structurally engineered permitting greater accessibility to the target region, e.g. the brain, than naturally occurring antibodies. Further, the subject chimeric monoclonal antibodies reproducibly bind to target cells.

The present invention provides a modified chimeric monoclonal antibody comprising two molecules of each of two different polypeptides. The shorter polypeptides function as the light chains of the antibody and the longer polypeptides function as the heavy chains of the antibody. Moreover, the polypeptide which functions as a heavy chain has a variable region characteristic of a first mammal and a constant region characteristic of a second mammal. Each polypeptide which functions as a light chain has a variable region characteristic of a mammal and a constant region characteristic of a mammal, wherein a receptor-binding ligand replaces at least a portion of the constant region of each of the polypeptides which function as the heavy chains of the antibody.

As used herein, modified chimeric monoclonal antibody means a genetically engineered protein comprising (1) four polypeptides, two of which are designated heavy chains and two of which are designated light chains; the

polypeptides encoded by a nucleic acid molecule having diverse genetic constitutions; and (2) altered at the constant region of the heavy chain.

As used herein, the heavy (H) and light (L) chains of the modified chimeric monoclonal antibody refer to the differences in the molecular weight of the polypeptides which compose the above-described antibodies.

Further, as used herein, the variable (V) region of the modified chimeric monoclonal antibody are the sequences on both the light- and heavy-chain located at the amino terminal end of the molecule.

Moreover, as used herein, the constant (C) region of the modified chimeric monoclonal antibody are the sequences on both the light- and heavy-chain at the carboxyl terminal portion of the antibody. Accordingly, the chains of the immunoglobulin molecule can be divided into their V and C components so that a light chain consists of two parts, i.e. V_L and C_L (variable $_{light}$ and constant $_{light}$). Similarly, the heavy chain can be divided into V_H and C_H (variable $_{heavy}$ and constant $_{heavy}$).

One embodiment of the present invention provides that the first mammal is mouse and the second mammal is human. In another embodiment, the first mammal is human and the second mammal is mouse. Moreover, also within the scope of this invention are chimeric monoclonal antibodies which comprise polypeptides from mammals such as rats, moles, shrews, monkeys, bats, hares, rabbits, dogs, cats, whales, dolphins, elephants, horses, cows, deers or any combination thereof.

Further, in accordance with the practice of the invention, the variable region and the constant region of the light chain are both characteristic of the second

mammal. Alternatively, the variable region and the constant region of the light chain are both characteristic of the first mammal. A further alternative provides that the variable region of the light chain is characteristic of either the first or the second mammal and the constant region of the light chain is characteristic of the other mammal.

Further, the subject invention provides that the receptor-binding ligand comprises a growth factor. Examples of growth factors include but are limited to insulin, insulin-like growth factor, platelet-derived growth factor, epidermal growth factor, transforming growth factor, nerve growth factor, and growth hormone.

As used herein, insulin means either (1) naturally-occurring insulin whether of human or animal origin or 2) biosynthetic insulin from human or animal whether produced by genetic engineering methods or otherwise.

Similarly, as used herein, insulin-like growth factor, platelet-derived growth factor, epidermal growth factor, transforming growth factor, nerve growth factor, and growth hormone means either the naturally-occurring or synthetic form thereof.

Further, examples of insulin-like growth factor include but are not limited to insulin growth factor 1 and insulin growth factor 2.

In the practice of the invention a growth hormone includes but is not limited to growth hormone releasing factor. Moreover, as used herein, examples of growth hormones include human, avian, equine, porcine, ovine, bovine, and piscine growth hormones.

Additionally, examples of transforming growth factor include but are not limited to transforming growth factor-α and transforming growth factor-ß, i.e. transforming growth factor-ß1, transforming growth factor-ß2, and transforming growth factor-ß3.

Additionally, in accordance with the practice of the invention, the receptor-binding ligand which replaces at least a portion of the constant region of the heavy chain polypeptides comprises tumor necrosis factor.

Alternatively, in another embodiment of the invention the receptor-binding ligand comprises transferrin. Further, in another embodiment of the invention the receptor-binding ligand comprises a lymphokine. Examples of suitable lymphokines are selected from a group consisting of macrophage inhibition factor, leukocyte migration inhibition factor, macrophage activating factor, macrophage cytotoxicity factor, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, lymphotoxin, monocyte-derived lymphocyte activating factor, and T helper cell replacing factor.

As used herein, a lymphokine means either (1) a naturally-occurring lymphokine whether of human or animal origin or (2) a biosynthetic lymphokine from human or animal whether produced by genetic engineering methods or otherwise.

Moreover, in the practice of this invention the antibody is selected from, but not limited to, any antibody from a group comprising an IgG, IgA, IgD, IgE or IgM antibody or any combination thereof.

Also, in accordance with the practice of the invention the variable region of the previously described chimeric

monoclonal antibody comprises immunoglobulin-like ligand binding regions having a folded configuration. Examples of immunoglobulin-like ligand binding regions include, but are not limited to, domains from T cell receptors, major histocompatibility complex antigens, CD4, and CD8.

In accordance with the practice of the invention, the variable region comprises the domain of a T cell receptor. Alternatively, in another embodiment, the variable region comprises the domain of a MHC antigen, e.g. an HLA antigen or an H-2 antigen. In a further embodiment, the variable region comprises the domain of a surface glycoprotein CD4. Moreover, in another embodiment the variable region comprises the domain of a surface glycoprotein CD8.

The CD4 molecule is a cell surface glycoprotein which interact with targets bearing class II major histocompatibility complex (MHC) molecules (73). CD4 acts as a recognition molecule mediating appropriate interactions between the CD4+ T lymphocytes and its target. Additionally, CD4 is the cell surface receptor for the AIDS virus. Accordingly, the subject chimeric monoclonal antibodies having a variable region of the heavy chain comprising the CD4 domain is important as a therapeutic and diagnostic agent in mediating T lymphocyte function and alleviating AIDS.

Additionally, CD8 is a cell surface glycoprotein which is found on a subpopulation of T lymphocytes, i.e. CD8+ T lymphocytes. CD8+ T lymphocytes interacts with cells expressing class I MHC molecules. Accordingly, monoclonal antibodies comprising CD8 inhibit T cell function by specifically binding with molecules which are directed against CD8. In this regard, the subject chimeric monoclonal antibody is important in mediating T lymphocyte function, specifically, the embodiment of the

invention, wherein the variable region of the heavy chain comprises the CD8 domain.

T cell receptors are special membrane-anchored cell surface proteins which are found on T cells and are analogous to antibodies. Moreover, T cell receptors are the mechanism by which T cells interact with antigen, thereby, allowing T cells to carry out cellular immune responses. In this regard, the subject chimeric monoclonal antibodies comprising a domain of the T cell receptor, compete with naturally-occurring T cell receptors for the antigen, thus, preventing T cells from mediating cellular immunity. This embodiment of the subject invention is useful in treating auto-immune diseases, e.g. lupus erythmatosus, AIDS.

As used herein, a major histocompatibility antigen means a transplantation antigen, i.e. of either mouse origin such as H-2 class proteins or of human origin such as HLA proteins. Transplantation antigens are structurally similar to antibody molecules.

Further, the subject chimeric monoclonal antibodies comprising a domain of MHC antigens are important in preventing rapid rejection of organ grafts between individuals by engaging in binding competition with transplantation antigens, i.e. HLA or H-2 antigens, thereby preventing rapid rejection of organ grafts between individuals.

Additionally, the present invention provides an immunologically reactive complex comprising two different polypeptides, the shorter of which functions as a light chain and the longer of which functions as a heavy chain. The polypeptide which functions as the heavy chain has a variable region characteristic of a first mammal and a constant region characteristic of a second mammal and the

polypeptide which functions as the light chain has a variable region characteristic of a mammal and a constant region characteristic of a second mammal. Moreover, a receptor-binding ligand replaces at least a portion of a constant region of one of the polypeptides.

As used herein an immunologically reactive complex is a biosynthetic complex and is produced by genetic engineering methods or otherwise.

Moreover, the immunologically reactive complex comprises a first mammal which is human and a second mammal which is mouse. Alternatively, the first mammal is mouse and the second mammal is human. Moreover, also within the scope of this invention are immunologically reactive complexes which comprise polypeptides from mammals such as rats, moles, shrews, monkeys, bats, sloths, hares, rabbits, dogs, cats, whales, dolphins, elephants, horses, cows, deers or any combination thereof.

Further, in one embodiment of the invention, the variable region and the constant region of the light chain of the immunologically reactive complex are both characteristic of the second mammal. Alternatively, the variable region and the constant region of the light chain are both characteristic of the first mammal. Another alternative provides that the variable region of the light chain is characteristic of either the first or the second mammal and the constant region of the light chain is characteristic of the other mammal. For example, if the variable region of the light chain is characteristic of the first mammal then the constant region of the light chain is characteristic of the second mammal.

The invention provides the immunologically reactive complex, wherein the receptor-binding ligand replaces at

least a portion of the constant region of the polypeptide which functions as the light chain. Alternatively, the receptor-binding ligand replaces at least a portion of the constant region of the polypeptide which functions as the heavy chain.

Also, in accordance with the invention the receptor-binding ligand of the immunologically reactive complex is a growth factor. Suitable examples of growth factors include, but are not limited to, insulin, insulin-like growth factor (insulin growth factor 1 or insulin growth factor 2), platelet-derived growth factor, epidermal growth factor, transforming growth factor (such as transforming growth factor-α, transforming growth factor-ß1, transforming growth factor-ß2, or transforming growth factor-ß3), nerve growth factor, growth hormone (such as growth hormone releasing factor), tumor necrosis factor, transferrin, and lymphokines (such as macrophage inhibition factor, leukocyte migration inhibition factor, macrophage activating factor, macrophage cytotoxicity factor, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, lymphotoxin, monocyte-derived lymphocyte activating factor, and T helper cell replacing factor).

Additionally, in accordance with the practice of the invention, the variable regions of the polypeptides of the immunologically reactive complex comprises domains of T cell receptors. Alternatively, the variable region of the polypeptides comprise domains of MHC antigens (such as an HLA antigen or an H-2 antigen). Further alternatively, the variable regions of the polypeptides comprise domains of surface glycoproteins CD4 or CD8.

Additionally, the present invention provides a chimeric polypeptide capable of functioning as a heavy chain of an antibody. The chimeric polypeptide comprises a variable

region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand replaces at least a portion of the constant region of the polypeptide. Alternatively, the receptor-binding ligand is joined to at least a portion of the constant region of the polypeptide.

Additionally, the present invention provides a chimeric polypeptide capable of functioning as a light chain of an antibody. The chimeric polypeptide comprises a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, and a receptor-binding ligand which replaces at least a portion of the constant region of the polypeptide. Alternatively, the receptor-binding ligand is joined to at least a portion of the constant region of the polypeptide.

As used herein the chimeric polypeptide is a biosynthetic polypeptide and is made by genetic engineering methods or otherwise.

In accordance with the practice of the invention, the first mammal is human and the second mammal is mouse. Alternatively, the first mammal is mouse and the second mammal is human. Moreover, also within the scope of this invention are chimeric polypeptides which comprise polypeptides from mammals such as rats, moles, shrews, monkeys, bats, sloths, hares, rabbits, dogs, cats, whales, dolphins, elephants, horses, cows, deers or any combination thereof.

Further, the present invention provides a nucleic acid molecule encoding the above-described chimeric polypeptide. Moreover, the present invention provides an expression vector for producing a chimeric polypeptide comprising a nucleic acid encoding the chimeric polypeptide and suitable regulatory elements positioned

within the vector so as to permit expression of the polypeptide in a suitable host.

It would be clear to those in the art that "suitable regulatory elements" would encompass the genetic elements that control gene expression, e.g. the origin of replication, promoter, and expression control sequences such as enhancers.

Further, the present invention provides the previously described modified human chimeric monoclonal antibody to which a moiety is attached, i.e. a drug or a detectable label, wherein attachment may be effected at the variable region of the molecule.

Moreover, examples of suitable drugs include but are not limited to a cytotoxic agent, e.g. methotrexate, decarbazine, toxins (such as ricin, diphtheria toxin, pseudomonas, exotoxin-A, abrin, supporin, and gelnoin), anti-infectious agents, anti-septic agents, and anti-metabolites.

As used herein ricin and abrin means ricin and abrin isolated from a wide variety of natural sources, e.g. seeds, or synthesized by genetic engineering methods or otherwise.

An example of a suitable anti-metabolite includes 5-iodo-2'-deoxyuridine (IUdR). As used herein an anti-metabolite encompasses any chemical which interferes with the replication of DNA.

Moreover, examples of suitable detectable labels include but are not limited to an enzyme, biotin, a fluorophore, a chromophore, a heavy metal, a paramagnetic isotope, or a radioisotope. By suitable detectable labels applicants

contemplate any label which would be conducive to the detection of a complex which are known in the art.

It would be clear to those skilled in the art that one method of attaching the subject chimeric monoclonal antibody to an enzyme, e.g. horseradish peroxidase, would be by a modification of the periodate method (74). Alternatively, it would be clear to those skilled in the art to attach biotin to the subject chimeric monoclonal antibodies by the method of Bayer et al. (75).

With regard to drug moieties, it would be clear to those skilled in the art that the drug may be bound to the variable region of the subject chimeric monoclonal antibodies.

Further, the present invention provides a pharmaceutical composition comprising the above-described chimeric monoclonal antibody, immunological complex or polypeptide to which a moiety is attached, i.e. a drug or a detectable label, in an amount sufficient to deliver an effective dose of the drug and a pharmaceutically acceptable carrier.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers. Such carriers are well known in the art and may include, but are in no way and are not intended to be limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solutions, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets, coated tablets, and capsules.

Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium

sterate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

In this method, the administration of the composition may be effected by any of the well known methods, including but not limited to intravenous, intramuscular, subcutaneous and oral administration.

Additionally, the present invention provides a method of producing the above-described modified chimeric monoclonal antibody. The method comprises: a) cotransfecting a suitable/nonantibody-producing host cell with two expression plasmids, (i) one of which encodes a polypeptide capable of functioning as the heavy chain of the antibody and having a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand replaces at least a portion of the constant region of the heavy chain polypeptide and (ii) the other of which encodes a polypeptide capable of functioning as the light chain of the antibody and having a variable region characteristic of a mammal and a constant region characteristic of a mammal; (b) treating the cotransfected host cell so as to effect expression of the polypeptides encoded by the plasmids and formation of the chimeric monoclonal antibody within the host cell and excretion into the culture medium of the antibody by the host cell; and (c) recovering the resulting excreted chimeric monoclonal antibody, from the culture medium.

Further, the present invention provides another method of producing the above-described modified chimeric monoclonal antibody. The method comprises: a) cotransfecting a suitable/nonantibody-producing host cell

with an expression plasmid which encodes (i) a polypeptide capable of functioning as the heavy chain of the antibody and having a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand replaces at least a portion of the constant region of the heavy chain polypeptide and (ii) a polypeptide capable of functioning as the light chain of the antibody and having both a variable region characteristic of a mammal and a constant region characteristic of a mammal; (b) treating the cotransfected host cell so as to effect expression of the polypeptides encoded by the plasmid and formation of the chimeric monoclonal antibody within the host cell and excretion into the culture medium of the antibody by the host cell; and (c) recovering the resulting excreted chimeric monoclonal antibody, from the culture medium.

One procedure for preparing monoclonal antibodies involves constructing separate light and heavy chain immunoglobulin gene transfection vectors which compatibly replicate and amplify in host cells (45). This means that each plasmid can be manipulated separately, but still be maintained together in the host cell, facilitating gene transfer of both transfection vectors into mammalian cells using protoplast fusion or electroporation methods. Each protoplast fusion event then delivers both vectors into the same mammalian cell.

As used herein, cotransfection means the essentially simultaneous insertion of both heavy and light chain genes, either by means of one or two expression vectors, into a suitable/nonantibody-producing host cell. It would be clear to those skilled in the art that a suitable/nonantibody-producing host cell would encompass any cell, both eucaryotic or procaryotic, capable of effecting expression of the polypeptides encoded by the plasmids and formation of the chimeric monoclonal

antibody within the host cell and excretion into the culture medium of the antibody by the host cell.

Applicants point out that it would be clear to those skilled in the art that the vectors herein comprise any vectors known in the art which are suitable for producing the modified chimeric monoclonal antibodies of this invention. Moreover, vectors as used herein comprise plasmids, viruses (including phage) and integratable DNA fragments, i.e. fragments that are integratable into the host genome by recombination. In one example of the present invention, the vector may be a plasmid which is cloned in a bacterial cell and integrates into the host cell genome upon cotransfection.

In one embodiment of the above-described method the suitable, nonantibody-producing host cell is a human cell, i.e. a myeloma cell. Alternatively, in another embodiment, the suitable, nonantibody-producing host cell is a murine cell. Moreover, although the preferred host cells are human or murine cells, in principle any higher eucaryotic cell is workable, whether from vertebrate or invertebrate culture.

As used herein "recovering the resulting excreted chimeric monoclonal antibody, from the culture medium" means any method, e.g. separation by immunoprecipitation, solvent fractionation, classical and high pressure liquid column chromatography, i.e. size exclusion, ion exchange, partition, and adsorption chromatography in normal and reverse phase, which are generally known and accepted by those in the art as a means to separate and isolate proteins.

The present invention provides a method of delivering a drug to a cell, e.g. a brain cell, an adipose cell, a blood cell, an epithelial cell, a muscle cell, a nerve

cell, or a leukemic cell, having a receptor for a growth factor on a surface. The method comprises contacting the cell with the chimeric monoclonal antibody, immunologically reactive complex, or chimeric polypeptide, to which a moiety is attached, i.e. a drug or a detectable label, wherein the receptor-binding ligand of the antibody, immunologically reactive complex, or chimeric polypeptide, comprises the growth factor which binds to the receptor so that the antibody binds to the cell and thereby delivers the drug to the cell.

As used herein, an adipose cell means those cells responsible for the production and storage of fat. Further, epithelial cells are cells which line the inner and outer surfaces of the body.

The chimeric monoclonal antibodies, immunologically reactive complexes, and chimeric polypeptides described herein bind to receptors so as to be transported across the BBB. Small foreign molecules introduced into the circulation rapidly distribute themselves throughout the body's extracellular fluids; however, they are generally unable to penetrate the tissues of the brain, i.e. the blood-brain barrier (BBB). The BBB is a functional barrier between the brain capillaries and the brain tissue which allows some substances from the blood to enter the brain rapidly while other substances either enter slowly or not at all. Further, the BBB effectively restricts transport between blood and the central nervous system of certain molecules; especially those which are water soluble, charged, and larger than about 200 daltons. The BBB has been found to function over all anatomical regions of the central nervous system, except for small areas around the pituitary stalk, the preoptic recess and the area postreme beneath the floor of the 4th ventricle. The basis for this

barrier appears to be embodied in the endothelial cells of the blood capillaries in the brain.

The BBB is not a fixed barrier. It can be influenced by the metabolic requirements of the brain, in addition to insults such as mechanical trauma, cerebral embolism, hypercapnia, hypoxia, extensive stress, radiation, electroconvulsive shock, explosive decompression, and various toxic substances. All of these conditions may alter the permeability of the barriers and, subsequently, the composition of the extracellular fluid.

Various substances are transported across the BBB either by passive diffusion or, more often, by a carrier-mediated or active form of transport. However, movement is limited; even the movement of water across the capillary wall is limited. Accordingly, the antibodies, immunologically reactive complexes, and chimeric polypeptides of this invention comprise receptor binding ligands which bind to receptor capable of facilitating transport across the BBB.

In the practice of the above-described method of delivering a drug to a cell, the cell is a brain cell and the growth factor, i.e.insulin-like growth factor 1, insulin-like growth factor 2, insulin, and transferrin, which upon binding to the receptor results in transport of the antibody, immunologically reactive complex, or chimeric polypeptide across the blood-brain barrier.

Moreover, in the above-described method, the brain cell is abnormal and associated with progressive dementia and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody, immunologically reactive complex, or chimeric polypeptide effective to halt the progressive dementia.

As used herein, progressive dementia is defined as the gradual deterioration or loss of intellectual faculties, reasoning, power, and memory. Alternatively, the brain cell is abnormal and associated with cerebral cortical atrophy and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody, immunologically reactive complex, or chimeric polypeptide effective to halt the cerebral cortical atrophy.

As used herein, cerebral cortical atrophy is a symptom typically characteristic of Alzheimer's disease.

In another embodiment, the brain cell is malignant and associated with neurosarcoma and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody, immunologically reactive complex, or chimeric polypeptide effective to halt the neurosarcoma.

Further, in yet another embodiment, the brain cell is malignant and associated with a carcinoma and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody, immunologically reactive complex, or chimeric polypeptide effective to halt the carcinoma.

Alternatively, in another embodiment, the brain cell is malignant and associated with a carcinosarcoma and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody, immunologically reactive complex, or chimeric polypeptide effective to halt the carcinosarcoma.

Moreover, in accordance with the practice of the invention, the brain cell is malignant and associated with a sarcoma and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody, immunologically reactive complex, or chimeric polypeptide effective to halt the sarcoma.

Further, the brain cell is malignant and associated with a carcinomatous cerebellar degeneration and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody, immunologically reactive complex, or chimeric polypeptide effective to halt the carcinomatous cerebellar degeneration. As used herein carcinomatous cerebellar degeneration means a nonmetastic carcinoma taking the form of a diffuse degeneration of the cerebellar cortex and deep cerebellar nuclei.

Additionally, in the practice of the above-described method of delivering a drug to a cell, the cell is a cell selected from, but is not limited to, a group including a blood cell, a muscle cell, a nerve cell, a bone cell, and an epithelia cell. Moreover, in accordance with the above-described method the cell is malignant and associated with a melanoma and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody effective to halt the melanoma.

Alternatively, in accordance with the above-described method the cell is malignant and associated with a breast cancer and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody, immunologically reactive complex, or chimeric

polypeptide effective to halt the breast cancer. Further, the cell is malignant and associated with a lymphoma and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody, immunologically reactive complex, or chimeric polypeptide effective to halt the lymphoma. Also in accordance with the above-described method the cell is malignant and associated with a carcinoma and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody, immunologically reactive complex, or chimeric polypeptide effective to halt the carcinoma. Additionally, in accordance with the above-described method the cell is malignant and associated with a sarcoma and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody, immunologically reactive complex, or chimeric polypeptide effective to halt the sarcoma.

The invention further provides a method of detecting a cell having a receptor for a growth factor on its surface which comprises contacting the cell with the chimeric monoclonal antibody, immunologically reactive complex, or chimeric polypeptide to which a detectable moiety is attached, wherein the receptor-binding ligand of the antibody, immunologically reactive complex, or chimeric polypeptide comprises the growth factor which binds to the receptor so that the antibody, immunologically reactive complex, or chimeric polypeptide binds to the cell and thereby detects the cell.

In one embodiment of the method of detecting a cell having a receptor for a growth fact on its surface, the cell is a brain cell and the growth factor upon binding

to the receptor results is transport of the antibody, immunologically reactive complex, or chimeric polypeptide across the blood-brain barrier. Examples of suitable growth factors is selected from the group consisting of insulin-like growth factor 1, insulin-like growth factor 2, insulin, and transferrin.

Further, in accordance with the practice of the invention, the brain cell is abnormal and associated with argyrophil plaque and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody, immunologically reactive complex, or chimeric polypeptide effective to permit detection of the plaque.

Alternatively, the brain cell is abnormal and associated with a brain tumor and the contacting with the chimeric antibody, immunologically reactive complex, or chimeric polypeptide comprises contacting the cell with an amount of the antibody, immunologically reactive complex, or chimeric polypeptide effective to permit detection of the tumor.

It would be clear to those skilled in the art that the detection is accomplished using methods which depend upon the identity of the detectable moiety but which are nevertheless well known. For example, when the detectable moiety is radioactive, a liquid scintillation counter is employed. Alternatively, radioactive labels are detected by radiography or other methods which detect radioactive decay after separating the unreacted detectable antibody. Moreover, when the moiety is an enzyme, e.g. horseradish peroxidase in a standard assay, a spectrophotometer is employed. Further, when the moiety is fluorescent, a fluorometer may be used, e.g. fluorescence activated cell sorting.

The present invention additionally provides a modified chimeric monoclonal antibody comprising two molecules of each of two different polypeptides, the shorter of which functions as the light chains of the antibody and the longer of which polypeptides function as the heavy chains of the antibody. Each polypeptide which functions as a heavy chain has a variable region characteristic of a first mammal and a constant region characteristic of a second mammal. Further, each polypeptide which functions as a light chain has a variable region characteristic of a mammal and a constant region characteristic of a mammal, wherein a receptor-binding ligand is covalently attached to the ends of the constant regions of each of the polypeptides which function as the heavy chains of the antibody.

Further, another immunologically reactive complex is provided. The complex comprises two different polypeptides, the shorter of which functions as a light chain and the longer of which functions as a heavy chain, the polypeptide which functions as the heavy chain having a variable region characteristic of a first mammal and a constant region characteristic of a second mammal and the polypeptide which functions as the light chain having a variable region characteristic of a mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand is covalently attached to the ends of a constant region of one of the polypeptides.

The invention also provides a chimeric polypeptide capable of functioning as a light chain of an antibody comprising a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand is covalently attached to the ends of a constant region of one of the polypeptides.

Further, also within the scope of the invention is a chimeric polypeptide capable of functioning as a heavy chain of an antibody comprising a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand is covalently attached to the constant region of the polypeptide.

In addition to the treatment of aberrant cells and infections in the brain which require the antibody, immunologically reactive complex, or chimeric polypeptide to cross the BBB, the subject chimeric monoclonal antibodies, immunologically reactive complex, or chimeric polypeptide are also of importance for the treatment of aberrant or infected cells in all other locations of the body. For example, the subject chimeric monoclonal antibodies with or without a covalently linked anti-metastatic agent is useful for the treatment of all forms of cancer such as leukemia, lymphomas, carcinomas, adenomas, and melanomas, that reside in any part of the body.

This invention is illustrated in the Experimental Details section which follows. This section is set forth to aid an understand of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow.

EXPERIMENTAL DETAILS

Materials and Methods

Cell Lines:

Human lymphoblasts, IM-9, which express IGF1 receptors on their surface (51), were obtained from American Type Culture Collection and maintained in RPMI 1640 medium with 10% fetal calf serum (Hyclone, Logan, UT). Human epidermoid carcinoma, KB, which expresses growth factor receptors on its surface (37) and myeloma cells, i.e. P3X63Ag8.653 cells, were cultured in Iscove's Modified Dulbecco's Medium (IMDM, GIBCO, Grand Island, NY) with 10% calf serum (Hyclone).

Characterization of the resulting monoclonal antibodies

Carotid artery injection technique: based on Journal of Clinical Investigation 74:745-752, 1984

Inject the ^{35}S-test compound and ^{3}HOH (a freely diffusible internal reference) into the common carotid artery of anesthetized adult rat (2-3 rats, male, Sprague-Dawley; 200-300 grams) or rabbits. Decapitate the rat (or rabbits) fifteen seconds after injection. Solubilize a sample of the injection solution and the hemisphere ipsilateral to the injection, in duplicate, in 1.5 ml soluene-350 at 50°C for 2 hours before double-isotope liquid model system. Use a similar protocol for rabbit.

Cerebral spinal fluid (CSF) collection: based on Endocrinology 133 (6):2299-2301, 1983

Collect samples of CSF from the cisterna magna. Anesthetize a few animals (rats and rabbits) and place in a Kopf stereotaxic apparatus with the ear bars raised

approximately 15 cm above the surface of the table. Place the incisor bar into lowest position so that the animal's head is maximally ventroflexed. Mount a 30-gauge needle connected to PE-10 tubing horizontally on the electrode carrier and direct the needle at the midline of the neck. Determine the dorsal ventral location of the cisterna magna by lowering the needle 6.3 to 6.8 cm (depending upon the size and strain of the animal) from the occipital crest. Advance the needle slowly through the incision in the skin and through the dorsal neck muscles while a slight suction is applied via a 1.0 ml syringe attached to the distal end of the tubing. When the needle penetrates the atlanto-occipital membrane and gains access to the cisterna magna, CSF flow will be initiated. Disconnect the syringe and collect the sample in a micropipette via gravity drainage. Collect a sample of from 50 to 200 μl in 30 minute period. At the completion of the CSF sampling procedure, take a 1.0 ml blood sample from the tail capillary plexus. The animal is killed following the procedure. The experiments on the transcytosis of the blood brain barrier and the determination of final location of recombinant molecules requires only a few animals (4-6).

__Determination of serum half-life__

Inject ^{35}S-labeled chimeric antibodies intravenously through the tail vein (5 mice/protein). Bleed the mice periodically from the retro-orbital sinus with heparinized 50 to 100 microliter capillary pipet. After the procedure, kill the mice and assay for the presence of ^{35}S-labeled chimeric antibodies.

__Isolation of endothelial cells__

Mince and homogenize cerebral cortices from anesthetized one-month-old Sprague-Dawley rats (2-3 rats) or rabbits

(1 rabbit) after decapitation to isolate endothelial cells.

Determination of final localization

Inject the purified chimeric fusion protein into the common carotid artery of the subject, i.e. rats or rabbits, as described in the section on carotid artery injection techniques. Decapitate subjects. Examine brain and organ tissue specimen for the presence of the chimeric fusion protein.

Anesthesia

To relieve pain/discomfort, administer: Ketamine (45 mg/kg) and xylazine (88 mg/kg) to rabbits. Administer Ketamine (87 mg/kg) and xylazine (13 mg/kg) to rats. Check the heartbeat and respiratory rate neurological reflexes, and the color of mucous membrane to confirm a successful anesthesia.

A successful euthanasia

To perform euthanasia, use carbon dioxide for rats and sodium pentobarbital (100 mg/kg) for rabbits. Kill mice by cervical dislocation, after ether anesthesia. To confirm a successful euthanasia, monitor the heartbeat.

EXAMPLE 1

In creating antibody molecules with improved functional properties, the constant region of the antibody was replaced with a growth factor. The VH, CH1, hinge and first amino acid of CH2 from a chimeric mouse/human IgG_3 anti-dansyl antibody was joined to a cDNA encoding rat Insulin-like Growth Factor 1 (IGF1) immediately 3' to the leader sequence of IGF1. The chimeric heavy chain was introduced along with an anti-dansyl specific chimeric κ light chain into the immunoglobulin non-producing myeloma P3X63Ag8.653. The immunoglobulin/non-immunoglobulin IgG_3-IGF1 chimeric protein was efficiently produced and secreted (up to 30 $\mu g/10^6$ cells/24 hours). The IgG_3-IGF1 chimeric proteins retain their specificity to the antigen dansyl and bind to the IGF1 receptors of human lymphoblast IM-9, albeit with reduced affinity. The chimeric proteins elicited some of the same biologic effects (increased glucose and amino acid uptake) in human epidermoid carcinoma KB cells as human IGF1, but with reduced specific activity.

The reduced affinity and biologic activity may result from (1) the presence of the unprocessed IGF1 moiety, (2) the large size of the IgG_3-IGF1 chimeric protein (160 KDa) compared to IGF1 (7 KDa) and (3) three amino acid substitutions in rat IGF1 compared to human IGF1 which may lead to decreased affinity for the human IGF1 receptor. Although the chimeric proteins were less effective on a molar basis than intact IGF2, they still exhibited the proper binding specificity and the ability to elicit the biologic effects associated with IGF1; thus, demonstrating a new family of immunotherapeutic molecules targeted to growth factor receptors.

Construction and Characterization of the IgG_3-IGF1 Chimeric Protein:

By site directed mutagenesis a unique restriction enzyme site (Pvu II) was generated at the 5' site of the CH2 domain of the mouse/human IgG_3 heavy chain gene (72) and at the 3' of the leader sequence of rat IGF1 cDNA. IGF1 and IgG_3 were joined using the Pvu II sites. The chimeric IgG_3-IGF1 heavy chain gene and the chimeric κ light chain gene were simultaneously transfected into P3X63Ag8.653 by protoplast fusion (45, 58). Then transfected cells were selected with G418 (GIBCO) at 1.0 mg/ml and screened by Enzyme-linked Immunosorbent Assay (ELISA) for transfectomas producing the chimeric protein (58). The IgG_3-IGF1 chimeric proteins biosynthetically labeled with ^{35}S-Methionine (Amersham, Arlington Heights, IL) were analyzed by Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) with/without 2-mercaptoethanol (Kodak, Rochester) NY). The IgG_3-IGF1 chimeric proteins were purified by affinity column as previously described (40). The purity of the IgG_3-IGF1 chimeric proteins were determined by silver staining gel (41) and Fast Performance Liquid Chromatography (FPLC, Pharmacia, Piscataway, NJ).

Receptor Binding Assays:

IM-9 cells were washed twice with 100 mM HEPES buffer containing 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 15mM Glucose, and 1% BSA, pH 7.5 (HEPES-BSA buffer), resuspended in HEPES-BSA buffer, and incubated for 1 hr at room temperature (36). Cells were counted and resuspended at a concentration of 4 - 6 x 10^7 cells/ml. A 50 μl aliquot of this cell suspension (2 - 3 x 106 cells) was removed and incubated with 50 μl of human IGF1 purchased from Amgen (Thousand Oaks, CA), IgG_3-IGF1, IgG_3 or buffer and 100 μl buffer containing 1 μl of ^{125}I-IGF1

(Amersham; 2.5 μCi ^{125}I-IGF1 in 2 ml Phosphate Buffered Saline) rotating at 15°C for 2 hrs. All samples were duplicated. The cells were pelleted by 10 sec centrifugation in a microcentrifuge. 100 μl of the supernatant (total 200 μl) was saved for determination of unbound counts (S) and the pellets (P) were washed with 0.5 ml ice-cold PBS and counted in a gamma-counter (Beckman Gamma 5500, Fullerton, CA). The bound counts were normalized by dividing by the total counts (2S + P). The percent inhibitions were calculated as:

$$[1-P/(2S + P)/C] \times 100\%$$

where C is the counts bound in the absence of a competitor.

<u>Determination of α - [1-^{14}C] - Aminoisobutyric Acid (AIB) and 2-Deoxy-D-[1-^{14}C] Glucose (2-DG) Uptake:</u>

KB cells (5 x 10^5 cells/ml) were grown to confluence in a 24 well plate (FALCON, Lincoln Park, NJ) and washed three times with IMDM. After incubation with IMDM without serum overnight at 37°C, cells were washed with HEPES-BSA buffer and 100 μl same buffer added. HEPES-BSA buffer without glucose was used for 2-DG uptake. A 100 μl aliquot of various concentrations of the test samples (IGF1, IgG_3-IGF1, or IgG_3) was added into each well and incubated at 37°C for 6 hrs. After incubation, 24 μM ^{14}C-AIB (Dupont, Boston, MA) or 15 μM ^{14}C-DG (Amersham) was added into each well (2). The plates were rapidly washed with ice-cold PBS after a 15 min incubation at 37°C and cells lysed with 300 μl 1 N NaOH. Aliquots were counted for ^{14}C and normalized to the amount of total protein determined by the BCA protocol (Pierce Chemical Co., Rockford, IL).

<u>Results</u>

<u>Construction of a hybrid gene between human IgG_3 and rat insulin-like factor 1 (IGF1)</u>

Experiments were undertaken to produce an antibody combining site joined to a growth factor so that the resulting molecule would retain its ability to bind to both antigen and the growth factor receptor. For the initial construction, rat insulin-like growth factor 1 (IGF1) was chosen as the growth factor. Mature IGF1 (a 70 amino acid protein) is formed from the IGF1 precursor (a 130 amino acid protein) through proteolytic processing of both its leader peptide and its carboxy terminus. The amino acid at which the leader peptide is cleaved is a convenient site for joining to the Ig molecule. Preferably, the Ig molecule is human IgG_3 which has an extended hinge region of 62 amino acids. Further, IGF1 was placed distal to the hinge region of human IgG3 thus producing a spacing which facilitates simultaneous antigen and receptor binding.

To facilitate construction of fused genes, a unique restriction site (PvuII) was generated by site directed mutagenesis at the 5' end of the leader sequence of the rat IGF1 cDNA (**Figure 1A**). These mutations were confirmed by sequencing. The human IgG_3 gene which contains C_H1, hinge and 4 basepairs of C_H2 exon was joined to the IGF1 gene which in turn contains 2 basepairs of leader sequence, exon 2, exon 3, and exon 5. A chimeric constant region gene was constructed with a human IgG^3/IGF-1 chimeric constant region gene joined to a mouse anti-dansyl (DNS) variable region gene cloned into a transfection vector pSV2 ΔHgpt (**Figure 1B**). The mouse/human κ light chain specific for dansyl is contained in pSV184 ΔH neo.

The strategy for the construction of IgG_3-IGF1 fusion gene was as follows (**Figure 1A**). The fourth base (C) of the

C_H2 domain in human IgG3 was mutated to G by site-directed mutagenesis, resulting in the introduction of an unique PvuII restriction enzyme site. In addition, a unique PvuII site was introduced in IGF1 by changing the last base (C) of the leader sequence (Leader Seq. or LS) of rat IGF1 cDNA to T (thymidine). Human IgG_3 and rat IGF1 cDNA digested with PvuII were ligated, resulting in an in-frame IgG_3-IGF1 fusion gene without any significant amino acid substitutions.

Figure 1B is a schematic representation of the transfection vectors, the genetically engineered IgG_3-IGF1 fusion gene, and a proposed chimeric antibody. The mouse-human κ light chain gene is cloned into pSV184 ΔH neo, which is derived from the pACYC184, and contains the pACYC origin of replication, a chloramphenicol resistant gene (CM^r) for selection in <u>E. coli</u> and the <u>neo</u> gene (the dotted box) with the SV40 early region promoter (the shaded box) for selection in eucaryotic cells. The mouse-human IgG_3/rat IGF 1 heavy chain genes is cloned into pSV2 ΔH <u>gpt</u> gene (the dotted box). Boxes in the chimeric genes represent exons. The thick black solid line and boxes represent DNA of mouse origin while the thin solid and open boxes represent human DNA segments. The shaded box in the heavy chain genes represents the rat IGF1 cDNA. The sites of cleavage by restriction endonucleases open triangle), BamHI (open circle) and Hind III (closed circle) are shown. The mouse-human IgG_3/rat IGF1 chimeric protein produced by expression of both transfection vectors is shown at the bottom of the panel B. The black region of the chimeric molecule represents the mouse variable region domains specific for the hapten dansyl, the open regions represent the constant domains of human κ and IgG_3, and the shaded region represents the rat IGF1.

Transfection of the chimeric IgG_3-IGF1 gene

These heavy and light chain vectors were transformed into E. coli HB101, and the bacteria containing both vectors were used to transfect a non-producing myeloma cell line (P3X63 Ag8.653) by protoplast fusion. Alternatively, transfection may be effected by electroporation or the standard calcium phosphate precipitation method.

Stable transfectant cells (transfectomas) were selected using G418 and the supernatants of stable transfectomas were tested for the presence of an antibody protein by an Enzyme Linked Immunosorbent Assay (ELISA) using anti-human κ chain antibody. Seventy-seven stable transfectomas secreting complete antibody molecules were identified and recovered for further characterization.

The frequency of the desired transfectomas was 1.54 x 10^6 recipient myeloma cells. These transfectomas secreted 0.5-30 μg of chimeric molecule 10^6 cells/24 hours. The production level of the immunoglobulin/non-immunoglobulin chimeric molecules is not different from that of wild type chimeric antibodies.

Characterization of the IgG_3-IGF1 chimeric protein

The secreted IgG_3-IGF1 chimeric proteins were biosynthetically labeled with ^{35}S-methionine and the labeled proteins were purified by immunoprecipitation using dansyl-BSA (dansyl; 5-dimethylamino naphthalene-1-sulfonyl chloride) conjugated Sepharose beads or a rabbit anti-human IgG Fab antiserum and IgG Sorb. Reactivity of the fusion proteins with dansyl-BSA demonstrated that they retained their ability to react with their specific antigen. SDS-PAGE analysis (**Figures 2A and 2B**) demonstrated that the stable transfectomas produce novel chimeric molecules. As expected, the size of these

chimeric molecules is smaller than that of a normal IgG_3 antibody. However, the secreted chimeric heavy chain appeared heterogeneous in size because the recipient cells partially process the IGF1 precursor to mature IGF1. The incomplete proteolytic processing at the carboxy terminal of IgG_3-IGF1 heavy chain results in two distinctive heavy chains; a processed and an unprocessed heavy chain (**Figure 2B**).

When the chimeric molecules assemble to form the novel H_2L_2 antibody molecule, assembly of two unprocessed heavy chains results in the highest molecular weight chimeric molecule (top band in **Figure 2A**) and assembly of two processed heavy chains results in the lowest molecular weight chimeric molecule (bottom band in **Figure 2A**). Assembly between a processed and an unprocessed heavy chain results in intermediate sized chimeric molecule (middle band in **Figure 2A**). The IgG_3-IGF1 chimeric proteins were recognized by anti-IGF1 antisera, demonstrating that the IGF1 in the fusion protein assumes a native configuration.

Figures 2A and 2B represent SDS-PAGE analysis of IgG_3-IGF1 chimeric protein secreted by transfectomas. The secreted IgG_3-IGF1 chimeric protein biosynthetically labeled with ^{35}S-methionine was analyzed under non-reducing (panel A) and reducing (panel B) conditions. The labeled chimeric protein was precipitated with either dansyl-Sepharose (DNS-Sepharose: left in panel A) or anti-human IgG Fab antisera/Staph A/IgG Sorb (a HUG/Fab-IgG-Sorb in panel A). The secreted IgG_3 chimeric antibody which is the basic structure of IgG_3-IGF1 chimeric protein is used as control. Under non-reducing conditions (Panel A) the three bands represent the heterogeneous assembly of the processed and unprocessed chimeric protein; under reducing conditions the processed and unprocessed chimeric protein are seen (Panel B).

IgG_3-IGF1 chimeric proteins were purified from culture supernatants using an affinity column with 2-dimethylamino naphthalene-5-sulfonyl chloride (MW 269; dansyl isomer) coupled to AH-Sepharose 4B. Bound protein was eluted with N-5-carboxypentyl-2-dimethylamino naphthalene-5-sulfonamide (MW 364; dansyl isomer) and free hapten removed by extensive dialysis. The concentrated purified proteins were tetrameric and heterogeneous in size as expected. No other protein was observed in silver stained gel (**Figure 3A**). When purified chimeric proteins were fractionated by Superose-12 chromatography, the three heterogeneous chimeric proteins were eluted in a broad peak between IgG1 chimeric antibody (murine variable region - human IgG1 constant region; M. W. 146 KDa) and IgG_3 chimeric antibody (murine variable region - human IgG_3 constant region; M.W. 170 KDa (**Figure 3B**). The approximate molecular weight of the $(IgG_3\text{-}IGF1)_2L_2$ chimeric protein is 160 KDa.

Figure 3A illustrates purified IgG3-IGF1 fusion protein fractionated by SDS-PAGE under non-reducing conditions and visualized using silver staining. **Figure 3b** is a chromatogram of the purified IgG_3-IGF1 chimeric protein fractionated using FPLC (Superose-12 column, flow rate:0.25 ml/min). The elution time of IgG_3-IGF1 chimeric protein is 42 min. The elution of time of mouse-human IgG_3 chimeric antibody (170 KDa) is 40 min and that of IgG1 chimeric antibody (146 KDa) is 45 min as indicated by arrows.

Binding of the IgG_3-IGF1 Chimeric Protein to the IGF1 Receptor

A critical attribute of the fusion protein is whether it retains its ability to bind to the IGF1 receptor. To assess this, unlabeled recombinant human IGF1, wild type chimeric IgG_3 and the IgG_3-IGF1 chimeric protein were used

to inhibit the binding of ^{125}I-IGF1 to human lymphoblast IM-9 cells (20). Both IGF1 and IgG_3-IGF1 inhibited the binding of ^{125}I-IGF1 in a dose dependent manner (**Figure 4**). The 50% inhibition of ^{125}I-IGF1 binding occurred at recombinant IGF1 concentration of 2.25×10^{-9} M and at a IgG_3-IGF1 chimeric protein concentration of 3.15×10^{-7} M. Therefore, the IgG_3-IGF1 chimeric protein was 0.7% as effective as recombinant human IGF1 in inhibiting ^{125}I-IGF1 binding. However, the wild type chimeric IgG_3 did not show any inhibition of ^{125}I-IGF1 binding, even at a concentration as high as 2.7×10^{-6} M. Therefore, the competition by the chimeric protein was a consequence of the presence of the IGF1 moiety.

Figure 4 is a graph showing competitive inhibition of binding of ^{125}I-IGF1 to IM-9 lymphocytes. Approximately 3×10^{-6} IM-9 cells were incubated at 15°C with a constant amount of ^{125}I-IGF1 and the indicated concentration of unlabeled competitors (recombinant IGF1, IgG3-IGF1 chimeric protein and IgG_3 chimeric antibody). After 2 hours of incubation, the amount of receptor-bound radioactivity was determined. Values are expressed as the relative inhibition of binding compared to using only labeled tracer ^{125}I-IGF1. Results shown for each curve are the mean of duplicate experiments.

The IgG_3-IGF1 chimeric proteins have been tested for binding to two different primary cultures of human brain endothelial cells; one at an early passage (≈13) and one at a later passage (≈22). Comparable levels of binding with both of these cell lines have been observed (data now shown). The IgG_3-IGF1 chimeric proteins bound to both these cell lines in a specific manner, but the control antibody, chimeric IgG_3, does not bind to these cells.

__IgG_3-IGF1 Chimeric Protein Stimulation of Hexose and Amino Acid Uptake__

In human epidermoid carcinoma KB cells, fluid-phase endocytosis and exocytosis are stimulated by growth hormones (Insulin, Insulin-like growth factor 1, and epidermal growth factor); these cells possess 7.5 x 10^4/cell Type I IGF receptors (13). Therefore, the ability of IgG_3-IGF1 chimeric protein to stimulate 2-Deoxy-D-Glucose (2-DG) and α-Aminoisobutyric acid (AIB) uptake (11) was investigated and compared to IGF1 and IgG_3. The dose-response relationships of IgG_3-IGF1 chimeric protein stimulation of 2-DG and AIB uptake in K cells are shown in **Figure 5**. Based on half-maximal effective concentration, the relative potencies of IGF1 and IgG_3-IGF1 were 200:1 for AIB uptake and 25 - 100:1 for 2-DG uptake. IgG_3 alone did not affect 2-DG and AIB uptake by KB cells. Therefore, the chimeric proteins exert the expected biological effects, but are less potent than the human IGF1 standard.

Figure 5 shows the relationship between IGF1 and IgG3-IGF1 and their stimulatory effects on AIB and 2-DG uptakes in κB cells. AIB (panel A) and 2-DG (panel B) uptakes were determined in the presence of various concentrations of IGF1, IgG3-IGF1, and IgG3 as control.

Discussion

Many problems have been encountered when investigators have attempted to use monoclonal antibodies as immunotherapeutic agents. Among the problems are efficiently targeting the antibodies to tumor cells while leaving normal cells untouched and gaining access to restricted locations in the body, e.g. the brain, lymph, liver, kidney, lung, adrenal, skin, and pancreas. Thus, the subject chimeric monoclonal antibody was constructed to overcome these problems. The chimeric protein was cotransfered together with a dansyl specific chimeric light chain and was efficiently produced and secreted (up

to 30 $\mu g/10^6$ cells/24 hrs) IgG_3-IGF1 chimeric proteins in a recipient non-producing murine myeloma. Thus high level expression of these recombinant molecules are feasible.

In this example, the constant region of the antibody was replaced with a growth factor e.g. insulin, IGF1, IGF2 and transferrin. The VH, CH1, hinge and first amino acid of CH2 from a chimeric mouse/human IgG_3 anti-dansyl antibody was joined to a cDNA encoding rat Insulin-like Growth Factor 1 (IGF1) immediately 3' to the leader sequence of IGF1. The chimeric heavy chain was introduced along with an anti-dansyl specific chimeric κ light chain into the immunoglobulin non-producing myeloma P3X63Ag8.653. The immunoglobulin/non-immunoglobulin IgG_3-IGF1 chimeric protein was efficiently produced and secreted (up to 30 $\mu g/10^6$ cells/24 hours). The IgG_3-IGF1 chimeric proteins retain their specificity to the antigen dansyl and bind to the IGF1 receptors of human lymphoblast IM-9, albeit with reduced affinity. The chimeric proteins elicited some of the same biologic effects (increased glucose and amino acid uptake) in human epidermoid carcinoma KB cells as human IGF1, but with reduced specific activity.

The reduced affinity and biologic activity may result from (1) the presence of the unprocessed IGF1 moiety, (2) the large size of the IgG_3-IGF1 chimeric protein (160 KDa) compared to IGF1 (7 KDa) and (3) three amino acid substitutions in rat IGF1 compared to human IGF1 which may lead to decreased affinity for the human IGF1 receptor. Although the chimeric proteins were less effective on a molar basis than intact IGF2, they still exhibited the proper binding specificity and the ability to elicit the biologic effects associated with IGF1; thus, demonstrating a new family of immunotherapeutic molecules targeted to growth factor receptors.

The novel IgG_3-IGF1 chimeric proteins retain their specificity for the antigen dansyl, the ability to bind to the IGF1 receptor with reduced affinity and the ability to exert some of the biologic effects of receptor binding. The reduced affinity for receptor and biologic activity may result from (1) the presence of the unprocessed IGF1 moiety thus resulting in a heterogenous population of IgG_3-IGF1 chimeric protein, (2) the large size of the IgG_3-IGF1 chimeric protein (160 KDa) compared to IGF1 (7 KDa) thus leading to decreased accessibility to the IGF1 receptor and decreased binding affinity; and (3) three amino acids substitutions in rat IGF1 compared to human IGF1 may lead to lower affinity for the human receptor.

In determining whether reduced affinity for receptor and biologic activity results from the presence of the unprocessed IGF1 moiety a second generation of proteins in which a stop codon is introduced at the end of the mature protein is engineered. Also, creating a size-reduced size Fab-IGF1 chimeric facilitates the determination of whether reduced affinity for receptor and biologic activity results from the large size of the IgG_3-IGF1 chimeric protein (160 KDa) compared to IGF1 (7 KDa). Moreover, altering three amino acids in rat IGF1 as compared to human IGF1 by site-directed mutagenesis additionally facilitates the determination of whether the reduced affinity is from the three amino acid substitutions.

The flexibility and accessibility of IGF1 also play an important role in the chimeric protein. The IGF1 moiety was disposed close to the carboxy terminal of the extended hinge of human IgG_3. IgG_3 is the most flexible human IgG (61). Accordingly, this flexibility optimize the ability to simultaneously bind the IGF1 receptor and antigen. However, the position of IGF1 immediately

carboxy terminal to the hinge brought the IGF1 molecules into contact with each other and interfered with their binding. The molecules are improved by placing the IGF1 on a ß-strand in C_H2 more distal to the hinge (9). Since the C_H2 domains do not normally contact each other this places the two IGF1 moieties at some distance from each other and thereby improves their binding efficiency.

The BBB in a normal brain effectively restricts transport between blood and the central nervous system of certain molecules, especially those which are water soluble, charged and larger than several hundred daltons (56). The IgG_3-IGF1 chimeric proteins bind specifically to the IGF1 receptor and to human brain endothelial cells (**Figure 11**). **Figure 11** is a line graph wherein the X axis is the time in hours after injection and the Y axis is the % injected dose of the IgG3-IGF1 chimeric construct. **Figure 11** shows that after injection in a rat animal model the IgG3-IGF1 chimeric construct was found and persisted in brain parenchyma cells compared to the capillary pellet. Accordingly, this chimeric construct crossed the BBB.

EXAMPLE 2

A. METHODS

Construction of chimeric fusion genes

Antibody molecules with a combining specificity, a growth factor to target to receptor-bearing cells, and immune effector functions contained in one molecule and antibody molecules with bifunctional combining specificities belong to a family of multi-functional chimeric antibodies which are directed to targeting therapy to malignant cells.

A suitable protocol to create a family of multi-functional chimeric antibodies is:

a. Create antibodies having a variable region from dansyl (DNS) specific hybridomas which have the advantage of high binding affinity. Alternatively, use anti-dextran variable (V) regions or domains having anti-tumor specificities.

b. Incorporate DNS-Cephalin into the cell membrane of eukaryotic cells so as to simulate cell surface markers (8).

c. Use the human constant regions of IgG_1 and IgG_3, both of which bind the high affinity Fc receptor and activate complement.

d. Use IGF1, IGF2, insulin and transferrin as replacements for at least a portion of the F_c region of the heavy chain (see Example 1).

e. Substitute IGF1 and insulin into the Fc of anti-DNS or anti-tumor chimeric IgG_3 to produce:

$$V_{\text{DNS/anti-tumor specificity}} - C_H1 - \text{hinge} - \text{IGF}$$

$$V_{\text{DNS/anti-tumor specificity}} - C_H1 - \text{hinge} - \text{insulin}$$

Preferably, use IgG_3 for the immunoglobulin sequences because of its extended hinge region allows the molecule greater flexibility and facilitates binding of the growth factor to its receptor.

Molecules capable of recruiting human effector functions are:

$$V_{DNS/anti\text{-}tumor\ specificity} - C_H1 - \text{hinge} - C_H2 - \text{ligand}$$
$$V_{DNS/anti\text{-}tumor\ specificity} - C_H1 - \text{hinge} - C_H2\ C_H3 - \text{ligand}$$

C_H2 contains the binding site for the high affinity Fc receptor and for Clq.

<u>Gene Transfection</u>

The modified transfection vectors developed by Oi and Morrison (45) were used. Insert the reconstructed chimeric Ig/receptor ligand gene into the transfection vector (pSV2ΔHgpt), a derivative of pSVgpt. pSV2ΔHgpt contains the pBR322 origin of replication and has an ampicillin resistant gene for selection in <u>E. coli</u> and the <u>gpt</u> gene for selection in eukaryotic cells.

Clone the mouse/human light chimeric gene into the light chain vector (pSV184ΔH <u>neo</u>), a derivative of the pACYC184 plasmid, which contains the pACYC origin of replication a chloramphenicol resistant gene for selection in <u>E. coli</u>, and the <u>neo</u> gene for selection in eukaryotic cells.

Transform these plasmids into <u>E. coli</u>. Select the desired bacteria with chloramphenicol and ampicillin and fuse selected bacteria with a nonproducing myeloma cell line by protoplast fusion (39,58). Alternatively, use electroporation or calcium phosphate precipitation to introduce DNA into myeloma cells (39, 58).

Isolate stable transfectomas using selectible drug markers. Screen the culture supernatant of stable transfectomas by ELISA using any of the antigen dansyl, anti-idiotypic antibody, human κ chain or anti-ligand antibodies to identify clones secreting high levels of the chimeric antibodies.

Analysis of Transfectomas.

Expression of chimeric genes require efficient transcription, proper RNA processing, and exportation from the nucleus, so that the mRNA is stable and translated, and that the protein product is properly processed through the cell and is secreted without being degraded in the cytoplasm or in the medium.

To study expression of transfected genes analyze the total RNA from stable transfectomas on Northern blots using probes specific for various regions of the transfected gene (mouse V_H probe, human IgG_3 C_H1 probe, and ligand probe).

Further, to analyze the cytoplasmic and secreted chimeric proteins label selected transfectomas with ^{35}S--methionine, after which purify the labeled proteins by immunoprecipitation using dansyl conjugated beads, anti-idiotypic antibody conjugated beads or anti-ligand antibody conjugated beads.

Analyze these precipitated proteins by SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions. Isolate large quantities of protein from either culture supernatants or from ascitic fluid of tumor bearing mice. Determine whether transfectomas remain tumorigenic.

Characterization of chimeric fusion molecules

a. Determination of Fc receptor binding. Use anti-dansyl chimeric fusion molecules in a hapten-enzyme binding assay (40). Briefly, this involves the following: incubating the chimeric fusion molecules at varying concentrations after allowing sufficient time for the antibody to bind to the Fc receptors with the human monocyte-like cell line U937, which bears high-affinity Fc receptors. Wash the cells. Incubate cells with dansyl conjugated with B-galactosidase. Allow the antigen-antibody reaction to reach equilibrium and remove the unbound antigen by centrifuging the cells through a sucrose pad. Spectrophotometrically, determine amount of bound IgG-ß-Gal after incubating the cells with the substrate o-nitrophenyl galactoside and measure the absorbance at 420 nm. Using Scatchard analysis determine the apparent association of the antibody for the Fc receptor and the number of receptors per cell. Iodinate chimeric proteins without a combining specificity for dansyl and either determine direct binding or assay ability of recombinant protein to inhibit binding.

b. Complement (C') activation. Assay complement activation according to Oi, et al. (44), using dansyl coupled to BSA as antigen (Ag) for anti-dansyl chimerics or appropriate antigen for chimerics of different specificity. Mix titered guinea pig complement, a fixed amount of chimeric fusion molecules (Ab) and serially diluted dansyl-BSA in U bottomed microtiter wells. Incubate mixture. After incubation, add hemolysin-sensitized and ^{51}Cr-radiolabeled red blood cells to mixture, continue incubation, and pellet unlysed red blood cells. Collect supernatants and count radioactivity in a scintillation counter. Complement consumption is calculated as:

[1-cpm of (Ag+Ab+C')/cpm of (Ab+C')] x 100%.

c. Clq binding assay. Coat flexible microtiter plates with dansyl-BSA then incubate with saturating amounts of anti-dansyl chimeric fusion molecules. For antibodies with different specificities use appropriate antigen to coat the microtiter plate. Wash plates and allow several dilutions of ^{125}I-labeled human Clq to bind the Ag-Ab complex. After washing, cut the wells out of the plates and counted in a gamma counter (62).

d. Sensitivity to proteases. Digest chimeric fusion molecules with a variety of proteases, e. g. papain, pronase, trypsin, or pepsin. Allow digestions to occur for varying lengths of time (from 4 hours up to 24 hours). Analyze the degree of digestion of the protease-treated chimeric fusion molecules using SDS-PAGE and determine the amount of color released by enzyme labeling by scanning the stained gel with a spectrophotometer.

e. Determination of serum half-life of chimeric fusion molecules. In order to understand how the immunoglobulin/non-immunoglobulin chimeric fusion molecules behave under in vivo conditions, compare the half-lives of the immunoglobulin/non-immunoglobulin molecules and the chimeric mouse-human immunoglobulin molecules to determine the role of the constant region in determining half-life (62).

Intravenously inject purified radioactive chimeric molecules through the mouse tail vein and periodically bleed the mice into heparinized capillary pipets. Using ^{35}S-methionine biosynthetically label the proteins so that the labeling procedure will not alter the structure of the protein and thereby affect its metabolism. Collect blood and determine the radioactivity both before and after immunoprecipitation. Calculate the serum half-life of each chimeric molecule.

Comparison of the Biological Function of Chimeric Fusion Molecules to Specific Antigen Associated Cell

To determine whether the molecules exert any biological effect, e.g. antibody-dependent cell-mediated cytotoxicity (ADCC) of target cells (57) couple the target cells, i.e. 3T3-L1, IM-9 and K-562, with DNS hapten using DNS-Cephalin (8). Label target cells with ^{51}Cr chromate. Obtain human peripheral blood mononuclear cells from healthy donors and prepare human peripheral blood mononuclear cells by centrifugation on Ficoll-Hypaque (22). Add cells to tissue culture dishes coated with autologous serum and incubate for 90 min at 37°C in a humidified atmosphere of 8% CO_2-air. Pool nonadherent cells, i.e. lymphocytes, and harvest adherent cells, i.e. monocytes. Add lymphocyte and monocyte suspensions to target cells coupled/uncoupled with DNS in several ratios of effector:target cell. Centrifuge suspension. After centrifugation at 600 xg for 3 min, remove buffy coat and incubate it for 4 hrs at 37°C in humidified atmosphere of 8% CO_2-air. After centrifugation at 600 xg for 10 min remove and count an aliquot of supernatant from each sample. Calculate the percentage specific ^{51}Cr release as:

Percent release = 100X[(test release) - (spontaneous release)]/[(total radioactivity) - (spontaneous release)].

These experiments indicate the relative effectiveness of multifunctional molecules in targeting cells for ADCC and the necessary modification for a more effective chimeric protein.

Binding of Chimeric Fusion Molecules to Ligand Receptors

Since IGF1 insulin and transferrin receptors were identified on brain blood vessels both *in vivo* (64) and

in vitro (16), isolated brain microvessels have been used as a model system to test for binding and internalization of those ligands (16, 17, 18, 49). However those receptors also occur on non-brain cells. In particular the cultured human lymphoblast cell line IM-9 expresses large numbers of IGF1 receptors (52). The adipose cell line 3T3-LI expresses large numbers of insulin receptors (50, 53, 54) and the human chronic myelogenous leukemia cell line K-562 expresses large numbers of transferrin receptors (63, 66).

Carry out initial binding studies (16) of chimeric fusion molecules onto receptors at 15°C with the cultured cell lines (IM-9, 3T3-LI, and K562) and purified radioactive chimeric molecules. Only a small amount of ligand bound to receptor is internalized at 15°C. To determine non-specific binding, prepare reactions identically except for the addition of a high concentration (100 μg/ml) of unlabeled ligands. Withdraw aliquots periodically, determine the radioactivity of the pellets, and assay the amount of protein in each pellet by the method of Lowry (34). Carry out a competitive displacement experiment with the purified chimeric molecules and the appropriate dilution of unlabeled ligands. Monitor ligand degradation by precipitation in 10% TCA. From all these experiments, analyze the specific binding of the ligand moiety of the chimeric fusion molecules to their receptors.

When necessary to isolate brain endothelial cells to study endothelial transcytosis, first produce microvessels and then separate the endothelial cells. Isolate brain microvessels from mouse, rat, and/or rabbit using mechanical homogenization (6, 48). These preparations do not always exclude trypan blue. These preparation are metabolically active and have been studied with respect to glucose, lactate, and fatty acid

metabolism <u>in vitro</u> (3, 60). To culture endothelial cells (7, 21, 29) clean cortical tissue from brain of meninges and superficial blood vessels, mince cortical tissue from brain to small cubes, incubate cubes in proper medium containing 0.5% dispase for 3 hours at 37°C, and collect endothelial cells by centrifugation at 1,000 g for 1 minute. Suspend the pellets in medium containing 13% dextran. Separate microvessels from other brain tissue by centrifugation of the suspension at 5,800 g for 10 minutes. To remove the basement membrane and most pericytes treat the microvessel to a further 9-12 hour treatment with collagenase/dispase in medium. Pellet microvessels at 1,000 g for 20 minutes, suspend microvessels in medium, and keep suspension in liquid nitrogen until use. Filter some of the microvessel suspension through a 250 μM nylon mesh to remove non-digested material. Add proper medium. Remove the top layer of these cells and pellet the remaining cells. Resuspend the pellet and refilter it through 30 μM nylon mesh. Retain the endothelial cell in the mesh. Collect the endothelial cells in a plastic tube containing 40 ml of PBS 1.0 mM Ca and 5% BSA. Allow the sample to stand. Remove the top 10 ml and centrifuge the remaining suspension. Plate the pellet containing endothelial cells into gelatin coated tissue culture plates. Identify these primary cultured cells by morphology and their positive reaction for Factor VIII antigen.

<u>**Processing of Bound Complexes**</u>

At 37°C most ligands bound to their receptor will be internalized. At 4°C very little internalization takes place. Assess the internalization (endocytosis) of the purified chimeric fusion molecules with an acid-wash technique (18, 27, 46, 49). Incubate the cultured cells with trace amounts of the purified radioactive chimeric fusion molecules at 37°C, withdraw aliquots, quickly

count the pellets and resuspend it in ice-cold pH 3 barbital buffer. Acid-wash the pellets and then recount and assay it for the amount of protein. Acid washing removes the bound but not internalized proteins. Analyze both bound and internalized proteins by SDS-PAGE to determine whether degradation had taken place.

To determine the externalization (exocytosis) of the purified radioactive chimeric fusion molecules, reincubate the cells, after internalization at 37°C, in the presence of unlabeled ligands, to allow the surface-bound chimeric fusion molecules to be internalized or displaced from their receptor. Wash and rewash the cells and the resuspend them in fresh assay buffer, containing unlabelled ligands to prevent rebinding and incubate at 37°C. Withdraw aliquots periodically and count and assay pellets for amount of protein. Assay externalized protein by SDS-PAGE to determine whether the protein is intact.

For transcytosis studies involving rabbits, mice, and rats. Use experimental protocol similar to those which have been described (17). Inject the purified radioactive (^{35}S-methionine) chimeric molecules through the carotid artery, and decapitate animals after 15 seconds, sufficient time for a single passage through the brain (17). Rapidly remove the ipsilateral hemisphere, homogenize it through a needle, dissolve, and simultaneously count the ipsilateral hemisphere for ^{35}S and ^{3}H. From these results, calculate the brain uptake index (BUI).

Further, procure the CSF from the subarachnoid space or the cisterna magna in anesthetized animals. Obtain blood from the same animals by cardiac puncture. Determine brain chimeric molecule levels by the method of Frank et al. (17). Determine the levels of chimeric fusion

molecules in the CSF and the blood by radioimmunoassay using dansyl hapten or anti-idiotypic antibody. BUI and chimeric molecule levels in serum, brain, and CSF is indicative of whether chimeric molecules pass through the blood-brain barrier in vivo.

Determination of the Chimeric Fusion Protein Distribution of Target Cell Lines with Isolated Subcellular Fractions

To examine the intracellular targeting site of chimeric fusion molecules, analyze several subcellular fractions. Prepare samples by sonication after internalization of ^{35}S- or ^{125}I-labeled chimeric fusion molecule into 3T3-LI, IM-9 and K-562 cells coupled with DNS. Perform differential centrifugation to obtain nuclear/plasma membrane (Nuc/Pm) fraction; Mitochondria (Mit) fraction, high density microsomes (H. Micro) fraction, low density microsomes (L. Micro) and the cytosolic (Cyto) fraction (10, 59) (**Figure 6**).

Count each fraction by TCA precipitation. Furthermore, analyze the chimeric fusion molecules by SDS-PAGE to determine the location of intracellular target sites and to determine whether any degradation has taken place. Analyze the purity of each fraction by determining the enzymes which were present. Use 5'-nucleotidase and adenylate cyclase as a plasma membrane marker, Rotenone-insensitive NADH-cytochrome c reductase and glucose-6-phosphate phosphatase as an endoplasmic reticulum marker in H. Micro, galactosyl transferase as a Golgi apparatus marker in L. Micro and citrate synthase as a mitochondrial marker.

An alternative approach to cellular fractionation is immunohistochemical analysis and electron microscopy in order to identify the subcellular localization of the chimeric fusion molecules (14).

EXAMPLE 3

The first two domains of CD4 have been joined to IGF1 (Figure 9). The fusion protein was synthesized and secreted.

Mutagenesis of CD4

Convenient sites were created in the CD4 cDNA to allow the two, NH2 terminal, extracellular domains of CD4 to be ligated into the proposed construct.

5' CD4

A Bal I site was created upstream of the translation start site.

```
                                             Met Asn Arg Gly
5'...TTC CTC CCT CGG CAA GGC CACA ATG AAC CGG GGA...3'
                      *
Primer 3'...GGA GCC GTA CCG GTGT TAC T...
                      Bal I
```

3' CD4

A Sca I site was created one base after the codon for Glutamine 114.

```
      Gln  Gln  Gly  Ser  Leu  Thr  Leu  Thr  Leu  Glu
5'...CAG  GGG  CAG  AGC  CTG  ACC  CTG  ACC  TTG  GAG
                    **    *
Ser  Pro
AGC  CCC...3'

Primer 3'...CCC  GTC  ATG  AAC  TGG  GAC  TG...5'
                      Sca I
```

The resultant CD4 fragment will contain the 23 amino acids of the leader peptide and the first 114 amino acids of the mature protein. One additional base pair at the 3' ends of this fragment will combine with two bases 5' for the mature IGF1 to encode a threonine between glutamine 114 of CD4 and glycine 1 of IGF1. The first codon of the C-terminal propeptide of IGF1 has been replaced with a stop codon.

Construction of CD4-IGF1

Following the mutagenesis in pBluescript of CD4 to create the 3' Sca I site, IGF1 was inserted into this plasmid between this Sca I site and a Bam HI site in the poly linker, thus removing all downstream regions of CD4 (**Figure 9**). The IGF1 insert contains the coding region from two base pairs before the first codon of the mature protein through a string of Adenines not present in the genomic gene, and linked to 600 bp of the 3' untranslated (UT) region of IgG3. This 3' region was included for the poly A signal it contains. Questions have arisen as to whether or not this region actually contains the poly A signal. Within the 3' UT region of IGF1 are the Poly A signal like sequences AATGAAA and AAGTAAA. These sequences seem to function as a Poly A signal in that the cDNA shows that the message was polyadenylated about 40 bases downstream. Also, the IgG3-IGF1 chimeric constructs contain only these signals and appear to be expressed normally.

The final construct encodes the 23 amino acid propeptide and first 114 residues of CD4, a threonine, and the entire 70 residues of mature IGF1.

The construct described above has been inserted into the vector pAG4235 immediately downstream of an IgG3 promoter. This plasmid also contains a heavy chain

enhancer as well as the pSV2-gpt selectable marker (**Figure 10**).

Example 4

An antibody has been produced in which transferrin is joined to a mouse/human IgG3 chimeric anti-dansyl heavy chain immediately following the hinge region (**Figure 10**). This fusion protein assembles with the light chain and is secreted as an H_2L_2 molecule.

Example 5

Human IL-2 has been cloned using PCR technology with IL-2 specific oligomers. Cloned IL-2 was joined to a mouse/human IgG chimeric anti-dansyl heavy chain immediately following the hinge region using the methods described herein (**Figure 7**). This fusion heavy chain assembles with light chain and is secreted as an H_2L_2 molecule. **Figure 8** shows that the IL-2 fusion protein, designated TU2 has activity. Culture supernatants from a murine myeloma cell line transfectant synthesizing this protein will support the growth of an IL-2 dependent T cell line. Culture supernatants from the un-transfected myeloma cell line or from the myeloma cell line transfected with a chimeric Ig lacking IL-2 failed to support growth.

References

1. Beck, D.W., Vinters, H.V., Hart, M.N., and Cancilla, P.A. (1984) J. Neuropathol. Exp. Neurol. 43: 211-224.

2. Bequinot, F. et al. (1985) J. Biol. Chem. 260(29):15892.

3. Betz, L.A., and Goldstein, G.W. (1981) J. Physiol. (London) 312:365-376.

4. Bleil, J., and Bretsher, M.D. (1982) Eur. Molec. Biol. Orig. J. 1:351.

5. Boulianne, G.L., et al. (1984) Nature (London) 312:643-646.

6. Bowman, P.O., et al. (1981) In vitro, 17:353-362.

7. Bowman, P.O., et al. (1982) In Vitro 18:626-632.

8. Bruggemann, M., et al. (1987) J. Exp. Med. 166:1351-1361.

9. Burton, D. R. (1987) In: Molecular Genetics of Immunoglobulin (Calabi, F. and Neuberger, M. S., Eds.) Ch. 1 Elsevier Science Publishers, B. V.

10. Cotgreave, I.A. et al. (1988) J. Biochem Biophys. Meth. 16:247-254.

11. Dautry-Varsal, A. et al. (1983) Proc. Natl. Acad. Sci. USA 80:2258. USA 80:2258, 1983.

12. Duncan, A.R. et al. (1988) Nature, 332:363.

13. Duncan, et al. (1988) Nature 332:738.

14. Forti, G. et al. (1989) J. Steroid Biochem. 321: 135-144.

15. Fishman, J. B. et al. (1987) J. Neurosci. Res. 18:299.

16. Frank, H.J.L. et al. (1981) Diabetes 30:757-761.

17. Frank, H.J.L. et al. (1985) Diabetes 34:728-733.

18. Frank, H.J.L. et al. (1986) Diabetes 35:654-661.

19. Frank, H.J.L., et al. (1986) Ann. Intern. Med. 105:82-95.

20. Gatter, K.C. et al. (1983) J. Clin. Path. 36:539.

21. Gordon, P. et al. (1983) In Vitro, 19: 661-671.

22. Hale, G. et al. (1983) Blood 62:873.

23. Harding, C. et al. (1983) J. Cell Biol., 97:329.

24. Hopkins, C.R. et al. and Trowbridge, I.S. (1983) J. Cell Biol., 97:508.

25. Houghton, A.N., and Scheinberg, D.A. (1986) Seminars in Oncology, 13(2):165-172.

26. Jeffries, W.A. et al. (1984) Nature (London) 312:162.

27. Jialal., I. et al. (1984) Diabetes 33:784-522. 33:784-800.

28. Jones, P.T. et al. (1986) Nature, 321:522.

29. Kern, P.A. et al. (1983) J. Clin. Invest. 71:1822-1829.

30. Klausner, R.D. et al. (1983) Proc. Natl. Acad. Sci. USA 80:2263.

31. Kohler, G., and Milstein, C. (1975) Nature (London) 256:485-486.

32. Liu, A.Y. et al. (1987) Proc. Natl. Acad. Sci. USA, 84:3439.

33. LoBuglio, A. F., et al. (1989) Proc. Natl. Acad. Sci. USA 86:4220.

34. Lowry, O.H. et al. (1951) J. Biol. Chem. 183:265.

35. Mendelsohn, J. (1988) J. Prog. Allergy 45:147.

36. Misra, P. et al. (1986) J. Clin. Endocrin. and Metab. 63(6):1400.

37. Miyata, Y. et al. (1988) Exp. Cell Res. 178:73.

38. Morrison, S.L. (1984) Proc. Natl. Acad. Sci. U.S.A., 84:6851.

39. Morrison, S.L. et al. (1987) Ann. N.Y. Acad. Sci. 507:187.

40. Morrison, S.L. et al. (1988) Clin. Chem. 34:1668.

41. Morrisey, J.H. (1981) Anal. Biochem. 117:307.

42. Neuberger, et al. (1984) <u>Nature</u> (London), <u>312</u>:604.

43. Neuberger, M.J. et al. (1985) <u>Nature</u> (London), <u>314</u>:268-270.

44. Oi, V.T., et al. (1984) <u>Nature</u> <u>307</u>:136-140.

45. Oi, V.T., and Morrison, S.L. (1986) <u>BioTechniques</u> <u>4</u>:214.

46. Olefsky, J.M., and Kao, M. (1982) <u>J. Biol. Chem</u>. <u>257</u>:8667-8673.

47. Itabor, E. et al. (1989) <u>Science</u>, <u>243</u>:51.

48. Pardridge, W.M. et al. (1985) <u>J</u>. <u>Neurochem</u>. <u>44</u>:1178-1184.

49. Pardridge, W.M. et al. (1985) <u>J</u>. <u>Neurochem</u>. <u>44</u>:1771-1778.

50. Reed, B.C. et al. (1977) <u>Natl. Acad. Sci. USA</u> <u>74</u>:4876-4880.

51. Riechmann, L. et al. <u>Nature</u> (1988) <u>332</u>:323.

52. Rosenfeld, R.G., and Hintz, R.L. (1980) <u>Endocrin</u>. <u>107</u> (6):1841-1848.

53. Rubin, C.S. et al. (1978) <u>J</u>. <u>Biol. Chem</u>. <u>253</u>:7570.

54. Salzman, A. et al. (1987) <u>Biochemistry</u> <u>23</u>:6555-6565.

55. Sauvage, C.A. et al. (1987) <u>Cancer Res</u>. <u>47</u>:747.

56. Shapiro, W. R. and Shapiro, J. R. (1986) Seminars in Oncology 13(1):56.

57. Shaw, D.R. et al. (1988) J. Biol. Response Modifiers, 7:204.

58. Shin, S.-U., and Morrison, S.L. (1989) Method in Enzym., 178:451

59. Simpson,I.A. et al. (1983) Biochemica et Biophysica Acta 763:393-407.

60. Spatz, M., et al. (1978) J. Brain Res., 151:619.

61. Tan, L. K. et al. (1989) Proc. Natl. Acad Sci. USA, in press.

62. Tao, M.-H., and Morrison, S.L. Manuscript (1989) J. Immunol., 143:2595.

63. Tsavaler, L. Stein et al. (1986) J. Cell. Phys. 128:1.

64. Van Houten, M. et al. (1979) Nature (London) 282:623.

65. Van Reswoude, J. et al. (1982) Proc. Natl. Acad. Sci. USA 79:6186.

66. Vodineliah, L. et al. (1983) Proc. Natl. Acad. Sci. USA 80:835-839.

67. Waldmann, T.A., Longo, D.L., Leonard, W.J., et al. (1985) Cancer Res. 45:595.

68. Waldmann, T.A. (1986) Science 232:727.

69. Weissman, A. M. et al. (1986) J. Cell Biol. 102:951.

70. Yamashiro, P.J. et al. (1984) Cell 37:789.

71. Yelton, D.E. (1981) Ann. Rev. Biochem., 50:657-680.

72. Zoller, M.J., and Smith, M. (1982) Nucl. Acids. Res., 10(20):6487-6500.

73. V. Oi and S. Morrison (1986) BioTechniques, 4:214.

73. P. J. Maddon et al. (1985) Cell, 42:93.

74. Wilson, M.B. and Nakane, P.K. (1978) Immunofluorescence and Related Staining Techniques (Elsevier/North Holland Biomedical Press, Amsterdam) 215.

75. Bayer et al. (1979) Methods in Enzymology, 62:308.

76. Yelton, D.E. et al. (1981) J. Exp. Med., 156:1151.

77. Zack, D.J., et al. (1981) J. Exp. Med.. 154:1554.

78. Dangl, J.L., et al. (1988) EMBO J., 7:1989.

What is claimed is:

1. A modified chimeric monoclonal antibody comprising two molecules of each of two different polypeptides, the shorter of which functions as the light chains of the antibody and the longer of which polypeptides function as the heavy chains of the antibody, each polypeptide which functions as a heavy chain having a variable region characteristic of a first mammal and a constant region characteristic of a second mammal and each polypeptide which functions as a light chain having a variable region characteristic of a mammal and a constant region characteristic of a mammal, wherein a receptor-binding ligand replaces at least a portion of the constant region of each of the polypeptides which function as the heavy chains of the antibody.

2. The modified chimeric monoclonal antibody of claim 1, wherein the variable region and the constant region of the light chain are both characteristic of the second mammal.

3. The modified chimeric monoclonal antibody of claim 1, wherein the variable region and the constant region of the light chain are both characteristic of the first mammal.

4. The modified chimeric monoclonal antibody of claim 1, wherein the variable region of the light chain is characteristic of either the first or the second mammal and the constant region of the light chain is characteristic of the other mammal.

5. The modified chimeric monoclonal antibody of claims 1, wherein the first mammal is mouse and the second mammal is human.

6. The modified chimeric monoclonal antibody of claims 1, wherein the first mammal is human and the second mammal is mouse.

7. The chimeric monoclonal antibody of claims 1, wherein the receptor-binding ligand comprises a growth factor.

8. The chimeric monoclonal antibody of claim 7, wherein the growth factor comprises insulin.

9. The chimeric monoclonal antibody of claim 7, wherein the growth factor comprises insulin-like growth factor.

10. The chimeric monoclonal antibody of claim 9, wherein the insulin-like growth factor comprises insulin growth factor 1.

11. The chimeric monoclonal antibody of claim 9, wherein the insulin-like growth factor comprises insulin growth factor 2.

12. The chimeric monoclonal antibody of claim 7, wherein the growth factor comprises platelet-derived growth factor.

13. The chimeric monoclonal antibody of claim 7, wherein the growth factor comprises epidermal growth factor.

14. The chimeric monoclonal antibody of claim 7, wherein the growth factor comprises transforming growth factor.

15. The chimeric monoclonal antibody of claim 14, wherein the transforming growth factor comprises transforming growth factor-α.

16. The chimeric monoclonal antibody of claim 14, wherein the transforming growth factor comprises a transforming growth factor-ß.

17. The chimeric monoclonal antibody of claim 16, wherein the transforming growth factor ß comprises transforming growth factor-ß1.

18. The chimeric monoclonal antibody of claim 16, wherein the transforming growth factor ß comprises transforming growth factor-ß2.

19. The chimeric monoclonal antibody of claim 16, wherein the transforming growth factor ß comprises transforming growth factor-ß3.

20. The chimeric monoclonal antibody of claim 7, wherein the growth factor comprises a nerve growth factor.

21. The chimeric monoclonal antibody of claim 7, wherein the growth factor comprises growth hormone.

22. The chimeric monoclonal antibody of claim 7, wherein the growth factor comprises a growth hormone releasing factor.

23. The chimeric monoclonal antibody of claim 1, wherein the receptor-binding ligand comprises tumor necrosis factor.

24. The chimeric monoclonal antibody of claim 1, wherein the receptor-binding ligand comprises transferrin.

25. The chimeric monoclonal antibody of claims 1, wherein the receptor-binding ligand comprises a lymphokine.

26. The chimeric monoclonal antibody of claim 25, wherein the lymphokine is selected from the group consisting of macrophage inhibition factor, leukocyte migration inhibition factor, macrophage activating factor, macrophage cytotoxicity factor, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, lymphotoxin, monocyte-derived lymphocyte activating factor, and T helper cell replacing factor.

27. The chimeric monoclonal antibody of claim 1, wherein the antibody is an IgG antibody.

28. The chimeric monoclonal antibody of claim 1, wherein the antibody is an IgA antibody.

29. The chimeric monoclonal antibody of claim 1, wherein the antibody is an IgD antibody.

30. The chimeric monoclonal antibody of claim 1, wherein the antibody is in IgE antibody.

31. The chimeric monoclonal antibody of claim 1, wherein the antibody is an IgM antibody.

32. The chimeric monoclonal antibody of claim 1, wherein the variable region of the polypeptides comprise a domain of T cell receptor.

33. The chimeric monoclonal antibody of claim 1, wherein the variable region of the polypeptides comprise a domain of a MHC antigen.

34. The chimeric monoclonal antibody of claim 33, wherein the MHC antigen is an HLA antigen.

35. The chimeric monoclonal antibody of claim 33, wherein the MHC antigen is an H-2 antigen.

36. The chimeric monoclonal antibody of claim 1, wherein the variable region of the polypeptides comprise a domain of a surface glycoproteins CD4.

37. The chimeric monoclonal antibody of claim 1, wherein the variable region of the polypeptides comprise a domain of surface glycoproteins CD8.

38. An immunologically reactive complex comprising two different polypeptides, the shorter of which functions as a light chain and the longer of which functions as a heavy chain, the polypeptide which functions as the heavy chain having a variable region characteristic of a first mammal and a constant region characteristic of a second mammal and the polypeptide which functions as the light chain having a variable region characteristic of a mammal and a constant region characteristic of a second mammal, wherein a

receptor-binding ligand replaces at least a portion of a constant region of one of the polypeptides.

39. The immunologically reactive complex of claim 38, wherein the first mammal is mouse and the second mammal is human.

40. The immunologically reactive complex of claim 38, wherein the first mammal is human and the second mammal is mouse.

41. An immunologically reactive complex of claim 38, wherein the variable region and the constant region of the light chain are both characteristic of the second mammal.

42. An immunologically reactive complex of claim 38, wherein the variable region and the constant region of the light chain are both characteristic of the first mammal.

43. An immunologically reactive complex of claim 38, wherein the variable region of the light chain is characteristic of either the first or the second mammal and the constant region of the light chain is characteristic of the other mammal.

44. An immunologically reactive complex of claim 38, wherein the receptor-binding ligand replaces at least a portion of the constant region of the polypeptide which functions as the light chain.

45. An immunologically reactive complex of claim 38, wherein the receptor-binding ligand replaces at

least a portion of the constant region of the polypeptide which functions as the heavy chain.

46. The immunologically reactive complex of claim 38, wherein the receptor-binding ligand comprises a growth factor.

47. The immunologically reactive complex of claim 46, wherein the growth factor comprises insulin.

48. The immunologically reactive complex of claim 46, wherein the growth factor comprises insulin-like growth factor.

49. The immunologically reactive complex of claim 48, wherein the insulin-like growth factor comprises insulin growth factor 1.

50. The immunologically reactive complex of claim 48, wherein the insulin-like growth factor comprises insulin growth factor 2.

51. The immunologically reactive complex of claim 38, wherein the growth factor comprises platelet-derived growth factor.

52. The immunologically reactive complex of claim 38, wherein the growth factor comprises epidermal growth factor.

53. The immunologically reactive complex of claim 38, wherein the growth factor comprises transforming growth factor.

54. The immunologically reactive complex of claim 53, wherein the transforming growth factor comprises transforming growth factor-α.

55. The immunologically reactive complex of claim 53, wherein the transforming growth factor comprises a transforming growth factor-β.

56. The immunologically reactive complex of claim 55, wherein the transforming growth factor β comprises transforming growth factor-β1.

57. The immunologically reactive complex of claim 55, wherein the transforming growth factor β comprises transforming growth factor-β2.

58. The immunologically reactive complex of claim 55, wherein the transforming growth factor β comprises transforming growth factor-β3.

59. The immunologically reactive complex of claim 38, wherein the growth factor comprises a nerve growth factor.

60. The immunologically reactive complex of claim 38, wherein the growth factor comprises growth hormone.

61. The immunologically reactive complex of claim 60, wherein the growth hormone comprises a growth hormone releasing factor.

62. The immunologically reactive complex of claim 38, wherein the receptor-binding ligand comprises tumor necrosis factor.

63. The immunologically reactive complex of claim 38, wherein the receptor-binding ligand comprises transferrin.

64. The immunologically reactive complex of claim 38, wherein the receptor-binding ligand comprises a lymphokine.

65. The immunologically reactive complex of claim 64, wherein the lymphokine is selected from the group consisting of macrophage inhibition factor, leukocyte migration inhibition factor, macrophage activating factor, macrophage cytotoxicity factor, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, lymphotoxin, monocyte-derived lymphocyte activating factor, and T helper cell replacing factor.

66. The immunologically reactive complex of claim 38, wherein the variable regions of the polypeptides comprise a domain of a T cell receptor.

67. The immunologically reactive complex of claim 38, wherein the variable region of the polypeptides comprise a domain of a MHC antigen.

68. The immunologically reactive complex of claim 67, wherein the MHC antigen is an HLA antigen.

69. The immunologically reactive complex of claim 67, wherein the MHC antigen is an H-2 antigen.

70. The immunologically reactive complex of claim 38, wherein the variable regions of the polypeptides comprise a domain of a surface glycoproteins CD4.

71. The immunologically reactive complex of claim 38, wherein the variable regions comprise a domain of a surface glycoproteins CD8.

72. A chimeric polypeptide capable of functioning as a heavy chain of an antibody comprising a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand replaces at least a portion of the constant region of the polypeptide.

73. A chimeric polypeptide capable of functioning as a light chain of an antibody comprising a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand replaces at least a portion of the constant region of the polypeptide.

74. The chimeric polypeptide of any of claims 72 or 73, wherein the first mammal is human and the second mammal is mouse.

75. The chimeric polypeptide of any of claims 72 or 73, wherein the first mammal is mouse and the second mammal is human.

76. A nucleic acid molecule encoding the chimeric polypeptide of any of claims 72 or 73.

77. An expression vector for producing the chimeric polypeptide of claims 72 or 73 comprising a nucleic acid encoding the chimeric polypeptide and suitable regulatory elements positioned

within the vector so as to permit expression of the polypeptide in a suitable host.

78. A modified chimeric monoclonal antibody of claim 1 to which a moiety is attached.

79. The chimeric monoclonal antibody of claim 78, wherein the moiety comprises a drug.

80. The chimeric monoclonal antibody of claim 79, wherein the drug is a cytotoxic agent.

81. The chimeric monoclonal antibody of claim 80, wherein the cytotoxic agent is methotrexate.

82. The chimeric monoclonal antibody of claim 80, wherein the cytotoxic agent is a toxin.

83. The modified chimeric monoclonal antibody of claim 78, wherein the moiety comprises a detectable label.

84. The chimeric monoclonal antibody of claim 83, wherein the detectable label is biotin, a fluorophore, a chromophore, a heavy metal, a paramagnetic isotope, or a radioisotope.

85. A pharmaceutical composition comprising a chimeric monoclonal antibody of claim 79 in an amount sufficient to deliver an effective dose of the drug and a pharmaceutically acceptable carrier.

86. A method of producing a modified chimeric monoclonal antibody which comprises:

a) cotransfecting a suitable/nonantibody-producing host cell with two expression plasmids, (i) one of which encodes a polypeptide capable of functioning as the heavy chain of the antibody and having a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand replaces at least a portion of the constant region of the heavy chain polypeptide and (ii) the other of which encodes a polypeptide capable of functioning as the light chain of the antibody and having a variable region characteristic of a mammal and a constant region characteristic of a mammal;

(b) treating the cotransfected host cell so as to effect expression of the polypeptides encoded by the plasmids and formation of the chimeric monoclonal antibody within the host cell and excretion into the culture medium of the antibody by the host cell; and

(c) recovering the resulting excreted chimeric monoclonal antibody, from the culture medium.

87. A method of producing a modified chimeric monoclonal antibody which comprises:

a) cotransfecting a suitable/nonantibody-producing host cell with an expression plasmid which encodes (i) a polypeptide capable of functioning as the heavy chain of the antibody and having a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand

replaces at least a portion of the constant region of the heavy chain polypeptide and (ii) a polypeptide capable of functioning as the light chain of the antibody and having both a variable region characteristic of a mammal and a constant region characteristic of a mammal;

(b) treating the cotransfected host cell so as to effect expression of the polypeptides encoded by the plasmid and formation of the chimeric monoclonal antibody within the host cell and excretion into the culture medium of the antibody by the host cell; and

(c) recovering the resulting excreted chimeric monoclonal antibody, from the culture medium.

88. The method of any of claims 86 or 87, wherein the suitable, nonantibody-producing host cell is a human cell.

89. The method of any of claims 86 or 87, wherein the suitable, nonantibody-producing host cell is a murine cell.

90. The method of claim 88, wherein the human cell is a myeloma cell.

91. A method of delivering a drug to a cell having a receptor for a growth factor on surface which comprises contacting the cell with the chimeric monoclonal antibody of claim 79, wherein the receptor-binding ligand of the antibody comprises the growth factor which binds to the receptor so that the antibody binds to the cell and thereby delivers the drug to the cell.

92. The method of claim 91, wherein the cell is a brain cell and wherein the growth factor upon binding to the receptor results in transport of the antibody across the blood-brain barrier.

93. The method of claim 91, wherein the cell is a blood cell.

94. The method of claim 91, wherein the cell is a muscle cell.

95. The method of claim 91, wherein the cell is a nerve cell.

96. The method of claim 91, wherein the cell is a bone cell.

97. The method of claim 91, wherein the cell is a epithelia cell.

98. The method of claim 91, wherein the growth factor is selected from the group consisting of insulin-like growth factor 1, insulin-like growth factor 2, insulin, and transferrin.

99. The method of claim 92, wherein the brain cell is abnormal and associated with progressive dementia and the contacting with the chimeric antibody comprises contacting the cell with an amount of the antibody effective to halt the progressive dementia.

100. The method of claim 92, wherein the brain cell is abnormal and associated with a cerebral cortical atrophy and the contacting with the chimeric antibody comprises contacting the cell with an

amount of the antibody effective to halt the cerebral cortical atrophy.

101. The method of claim 92, wherein the brain cell is malignant and associated with a neurosarcoma and the contacting with the chimeric antibody comprises contacting the cell with an amount of the antibody effective to halt the neurosarcoma.

102. The method of claim 92, wherein the brain cell is malignant and associated with a lymphoma and the contacting with the chimeric antibody comprises contacting the cell with an amount of the antibody effective to halt the lymphoma.

103. The method of claim 92, wherein the brain cell is malignant and associated with a carcinosarcoma and the contacting with the chimeric antibody comprises contacting the cell with an amount of the antibody effective to halt the carcinosarcoma.

104. The method of claim 92, wherein the brain cell is malignant and associated with a sarcoma and the contacting with the chimeric antibody comprises contacting the cell with an amount of the antibody effective to halt the sarcoma.

105. The method of claim 92, wherein the brain cell is malignant and associated with a lymphoma and the contacting with the chimeric antibody comprises contacting the cell with an amount of the antibody effective to halt the lymphoma.

106. The method of claim 92, wherein the brain cell is malignant and associated with a carcinomatous

cerebellar degeneration and the contacting with the chimeric antibody comprises contacting the cell with an amount of the antibody effective to halt the carcinomatous cerebellar degeneration.

107. A method of any of claims 93, 94, 95, 96, or 97, wherein the cell is malignant and associated with a melanoma and the contacting with the chimeric antibody comprises contacting the cell with an amount of the antibody effective to halt the melanoma.

108. A method of any of claims 93, 94, 95, 96, or 97, wherein the cell is malignant and associated with a breast cancer and the contacting with the chimeric antibody comprises contacting the cell with an amount of the antibody effective to halt the breast cancer.

109. A method of any of claims 93, 94, 95, 96, or 97, wherein the cell is malignant and associated with a lymphoma and the contacting with the chimeric antibody comprises contacting the cell with an amount of the antibody effective to halt the lymphoma.

110. A method of any of claims 93, 94, 95, 96, or 97, wherein the cell is malignant and associated with a carcinoma and the contacting with the chimeric antibody comprises contacting the cell with an amount of the antibody effective to halt the carcinoma.

111. A method of any of claims 93, 94, 95, 96, or 97, wherein the cell is malignant and associated with a sarcoma and the contacting with the chimeric

antibody comprises contacting the cell with an amount of the antibody effective to halt the sarcoma.

112. A method of detecting a cell having a receptor for a growth factor on its surface which comprises contacting the cell with the chimeric monoclonal antibody of claim 83, wherein the receptor-binding ligand of the antibody comprises the growth factor which binds to the receptor so that the antibody binds to the cell and thereby detects the cell.

113. The method of claim 112, wherein the cell is a brain cell and wherein the growth factor upon binding to the receptor results is transport of the antibody across the blood-brain barrier.

114. The method of claim 112, wherein the growth factor is selected from the group consisting of insulin-like growth factor 1, insulin-like growth factor 2, insulin, and transferrin.

115. The method of claim 112, wherein the brain cell is abnormal and associated with an argyrophil plaque and the contacting with the chimeric antibody comprises contacting the cell with an amount of the antibody effective to permit detection of the plaque.

116. The method of claim 112, wherein the brain cell is abnormal and associated with a brain tumor and the contacting with the chimeric antibody comprises contacting the cell with an amount of the antibody effective to permit detection of the tumor.

117. The method of claims 91 or 112, wherein the cell is an adipose cell.

118. The method of claims 91 or 112, wherein the cell is an chronic myelogenous leukemia cell.

119. A modified chimeric monoclonal antibody comprising two molecules of each of two different polypeptides, the shorter of which functions as the light chains of the antibody and the longer of which polypeptides function as the heavy chains of the antibody, each polypeptide which functions as a heavy chain having a variable region characteristic of a first mammal and a constant region characteristic of a second mammal and each polypeptide which functions as a light chain having a variable region characteristic of a mammal and a constant region characteristic of a mammal, wherein a receptor-binding ligand is covalently attached to the ends of the constant regions of each of the polypeptides which function as the heavy chains of the antibody.

120. An immunologically reactive complex comprising two different polypeptides, the shorter of which functions as a light chain and the longer of which functions as a heavy chain, the polypeptide which functions as the heavy chain having a variable region characteristic of a first mammal and a constant region characteristic of a second mammal and the polypeptide which functions as the light chain having a variable region characteristic of a mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand is covalently attached to

the ends of a constant region of one of the polypeptides.

121. A chimeric polypeptide capable of functioning as a heavy chain of an antibody comprising a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand is covalently attached to the constant region of the polypeptide.

122. A chimeric polypeptide capable of functioning as a light chain of an antibody comprising a variable region characteristic of a first mammal and a constant region characteristic of a second mammal, wherein a receptor-binding ligand is covalently attached to the constant region of the polypeptide.

FIGURE 1(A)

HUMAN IgG3 CH2

```
        ←———IVS——|——CH2————→
WT:  TCTTCCTCA   GCA  CCT  GAA  CTC  CTG
                 Ala  Pro  Glu  Leu  Leu
                        ↓
        ←———IVS——|——CH2————→
MT:  TCTTCCTCA   GCA  GCT  GAA  CTC  CTG
                   ————————
                        PvuII
```

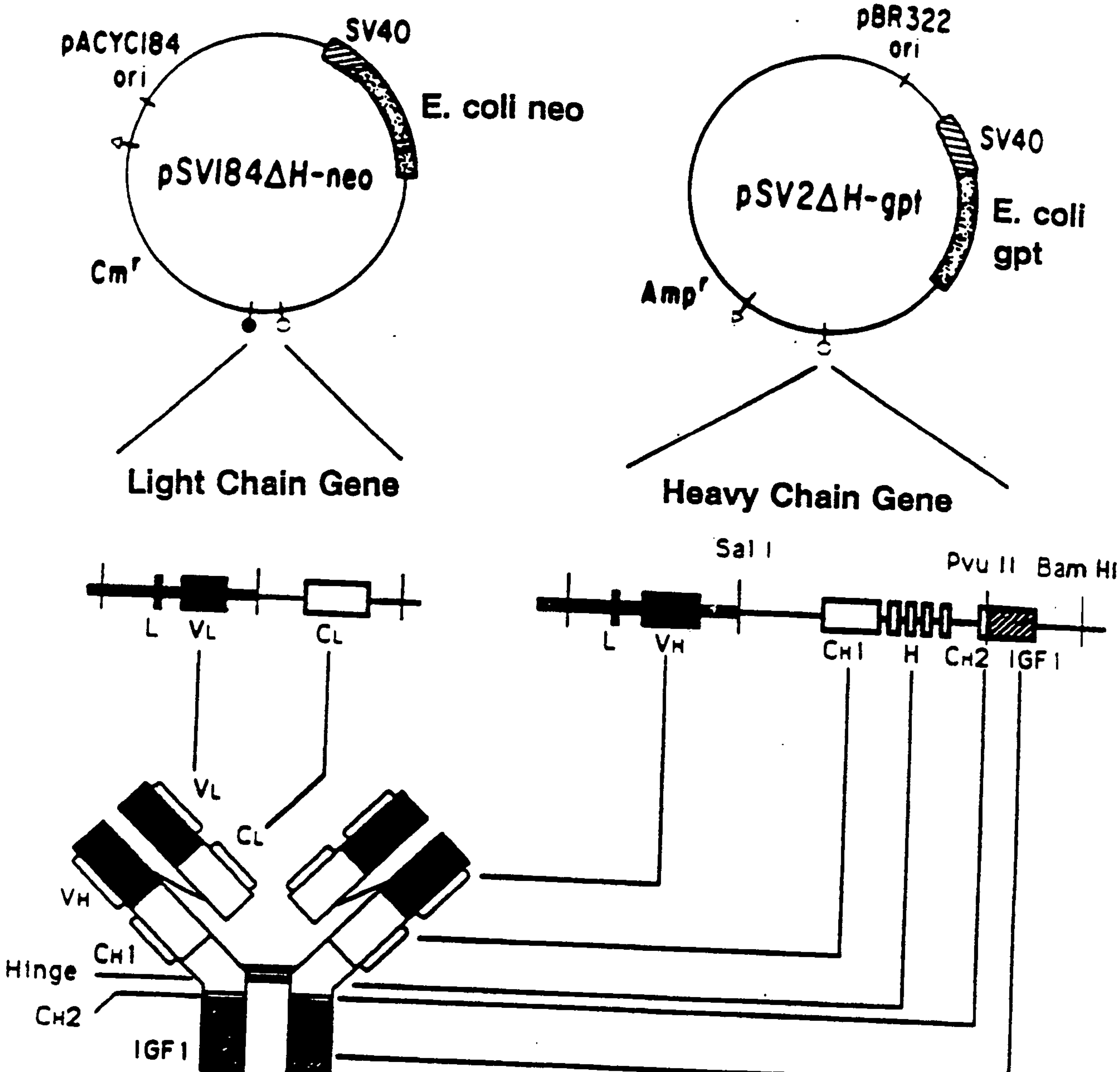
Rat IGF1 cDNA

```
      ←———Leader Seq——|—Exon2———→
WT:  TCG  GCC  ACA  GCC  GGA  CCA  GAG  ACC
     Ser  Ala  Thr  Ala  Gly  Pro  Glu  Thr
                     ↓
      ←———Leader Seq——|—Exon2———→
MT:  TCG  GCC  ACA  GCT  GGA  CCA  GAG  ACC
               ————————
                    PvuII
```

IgG3-IGF1 Fusion Gene

```
←—IVS————|—CH2—|LS|————————Exon2———→
TCTTCCTCA GCA  GCT  GGA  CCA  GAG  ACC
          Ala  Ala  Gly  Pro  Glu  Thr
```

FIGURE 1(B)



pACYC184
ori
SV40
E. coli neo
pSV184ΔH-neo
Cm^r
Light Chain Gene
pBR322
ori
SV40
E. coli
gpt
pSV2ΔH-gpt
Amp^r
Heavy Chain Gene
L
VL
CL
Sal I
Pvu II
Bam HI
L
VH
CH1
H
CH2
IGF1
VL
CL
VH
CH1
Hinge
CH2
IGF1

FIGURE 2(A)

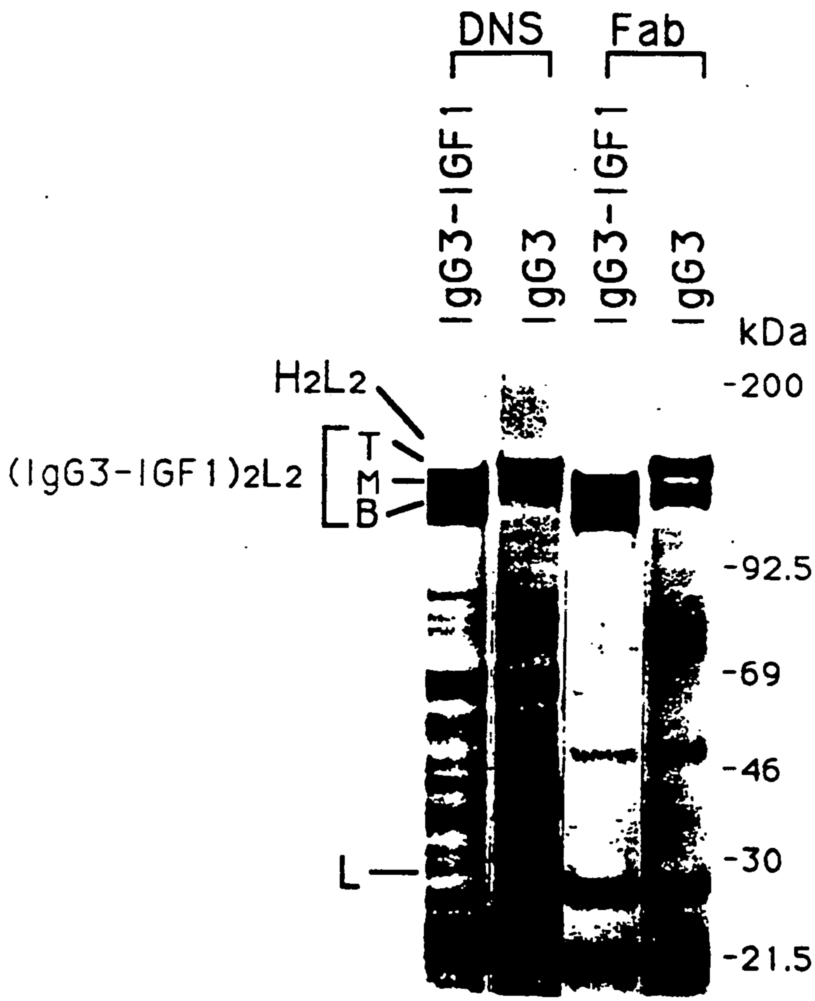

DNS
Fab
IgG3-IGF1
IgG3
IgG3-IGF1
IgG3
kDa
H2L2
T
(IgG3-IGF1)2L2
M
B
L
-200
-92.5
-69
-46
-30
-21.5

FIGURE 2(B)

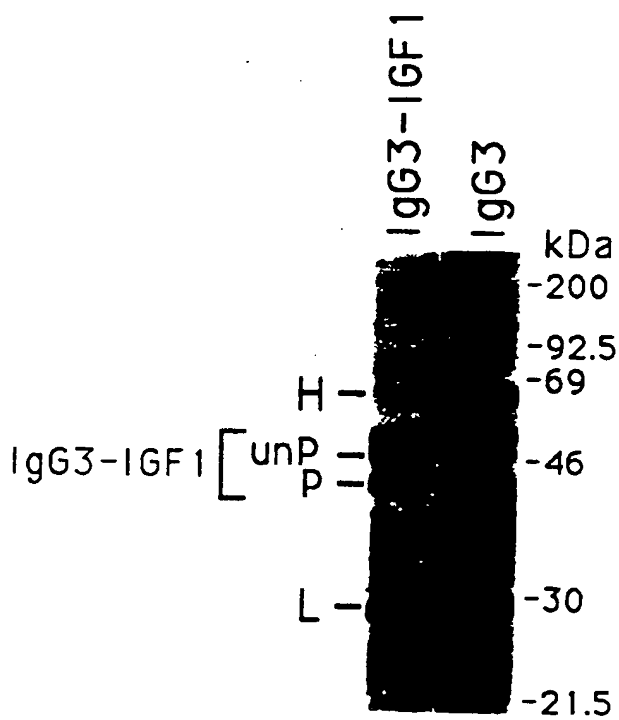


FIGURE 2(C)

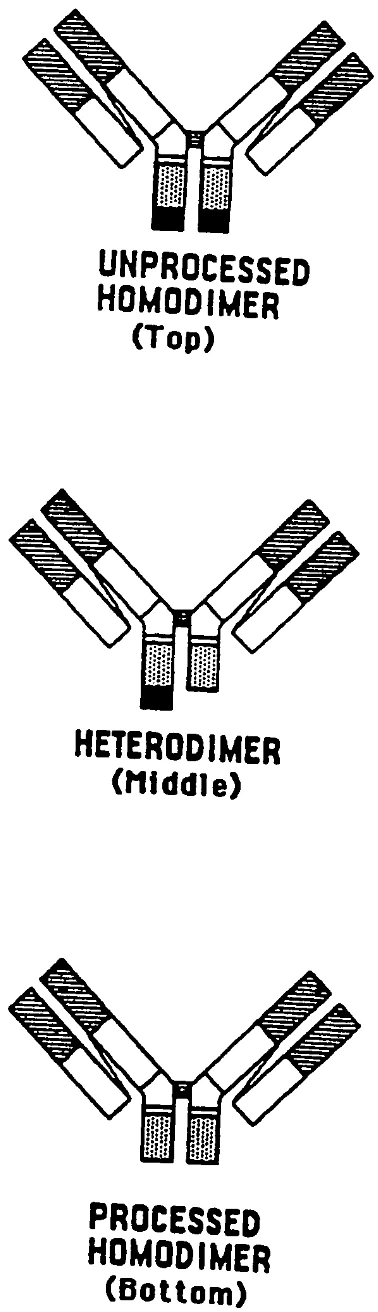

UNPROCESSED
HOMODIMER
(Top)
HETERODIMER
(Middle)
PROCESSED
HOMODIMER
(Bottom)

FIGURE 3(A)

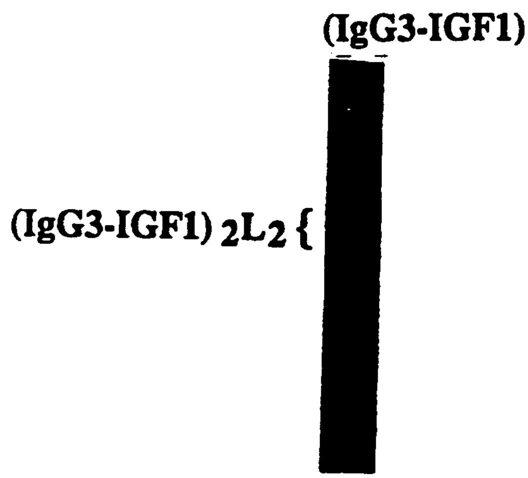
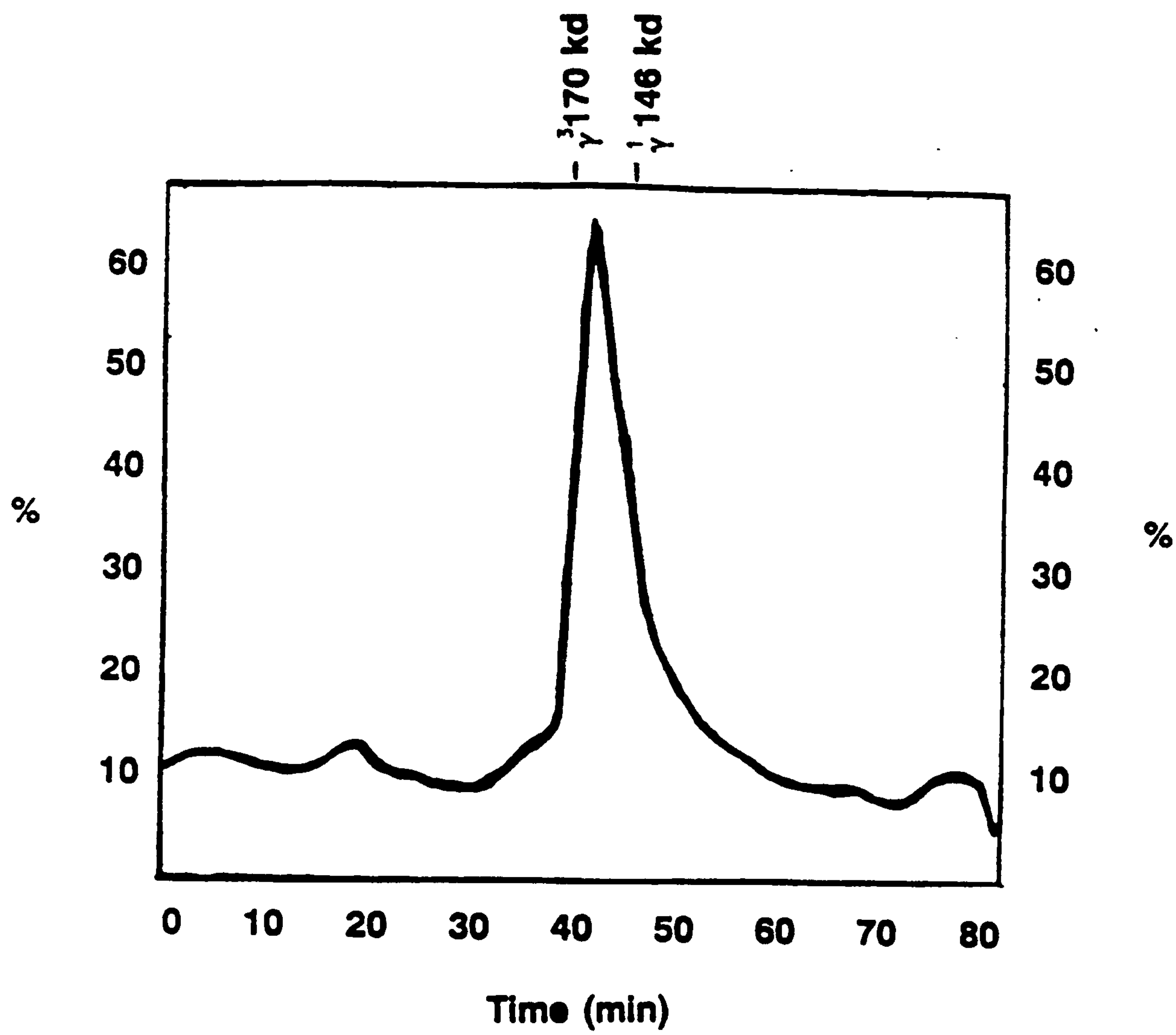


FIGURE 3(B)



γ^3 170 kd
γ^1 146 kd
%
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
Time (min)

FIGURE 4

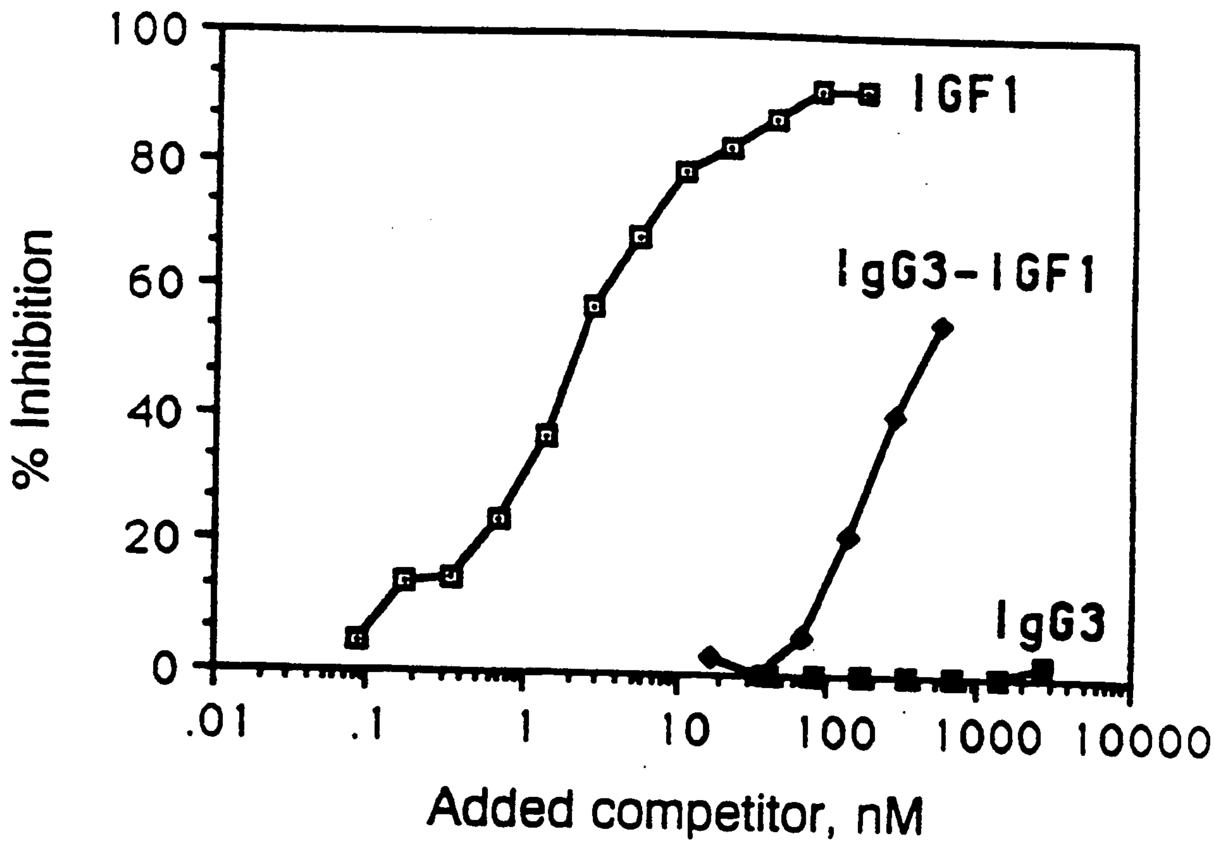

100
80
60
40
20
0
% Inhibition
IGF1
IgG3-IGF1
IgG3
.01
.1
1
10
100
1000
10000
Added competitor, nM

FIGURE 5(A)

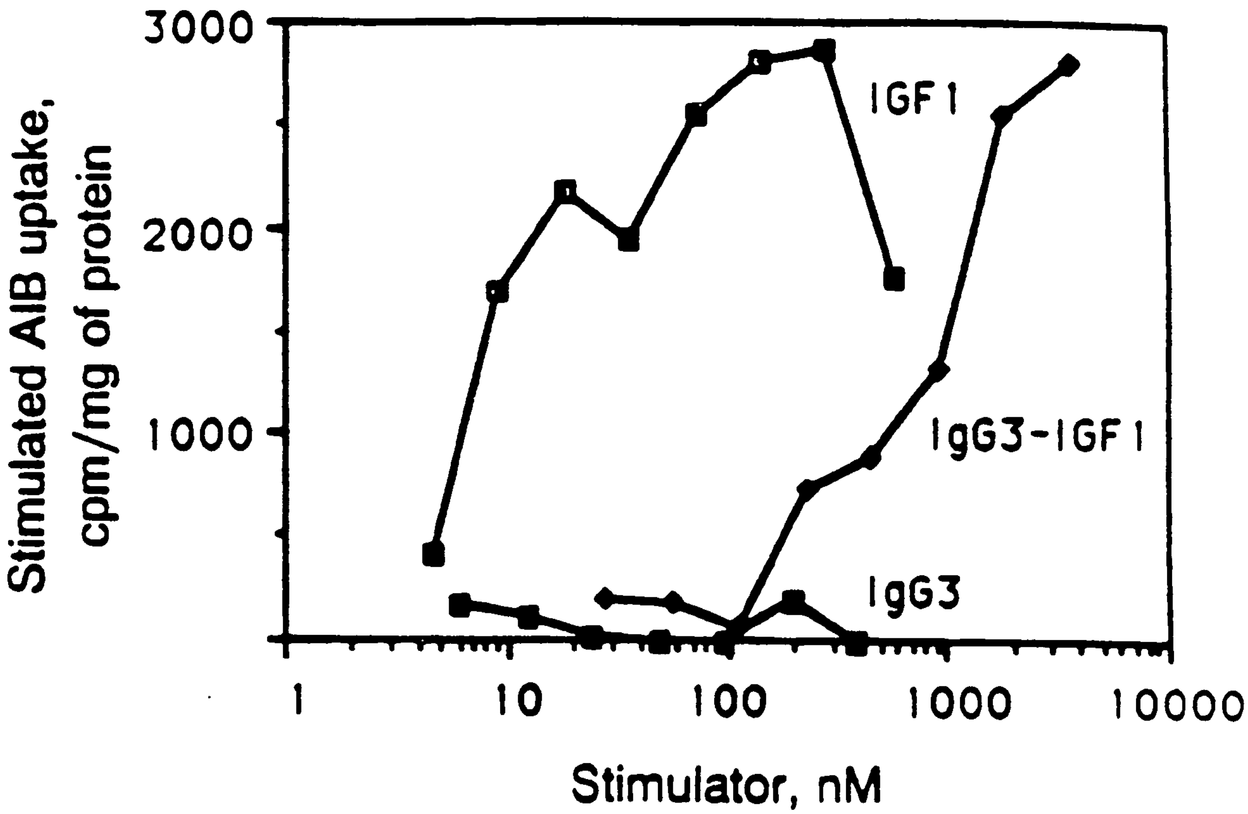

3000
2000
1000
Stimulated AIB uptake, cpm/mg of protein
1
10
100
1000
10000
Stimulator, nM
IGF1
IgG3-IGF1
IgG3

FIGURE 5(B)

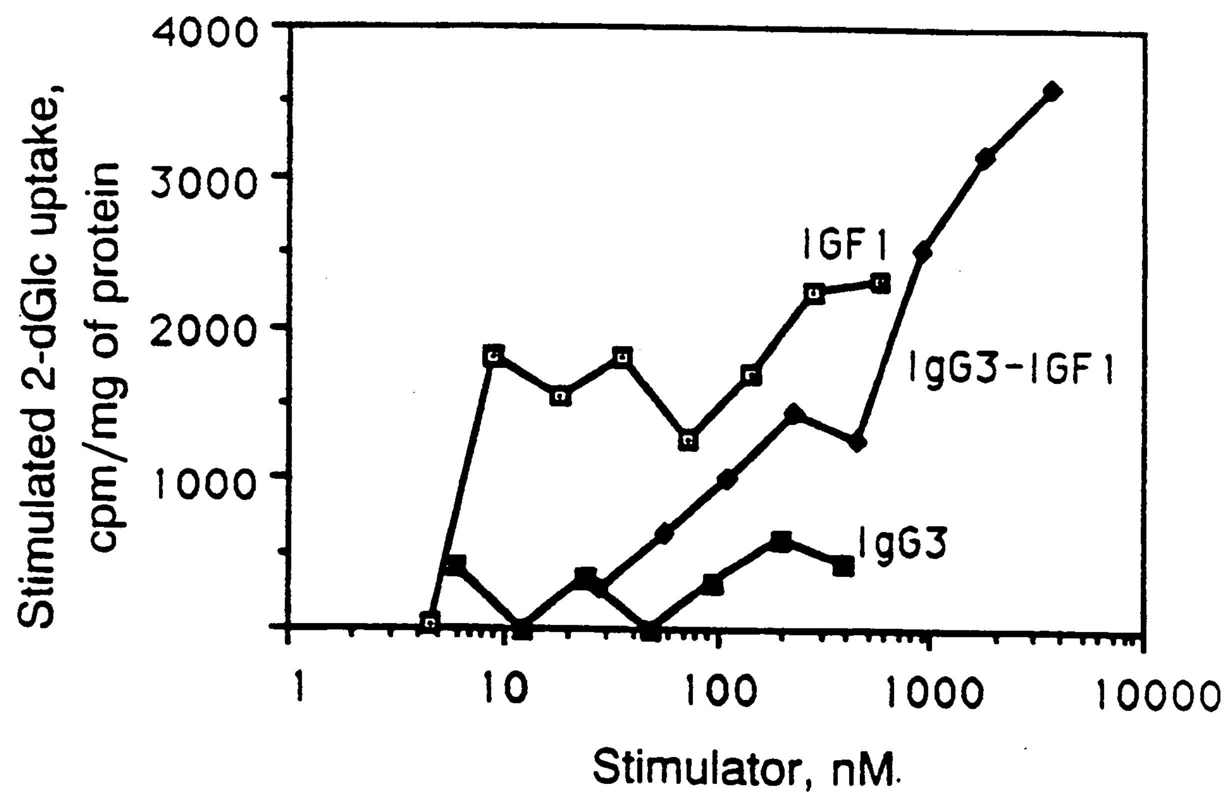

4000
3000
2000
1000
Stimulated 2-dGlc uptake, cpm/mg of protein
IGF1
IgG3-IGF1
IgG3
1
10
100
1000
10000
Stimulator, nM.

FIGURE 6

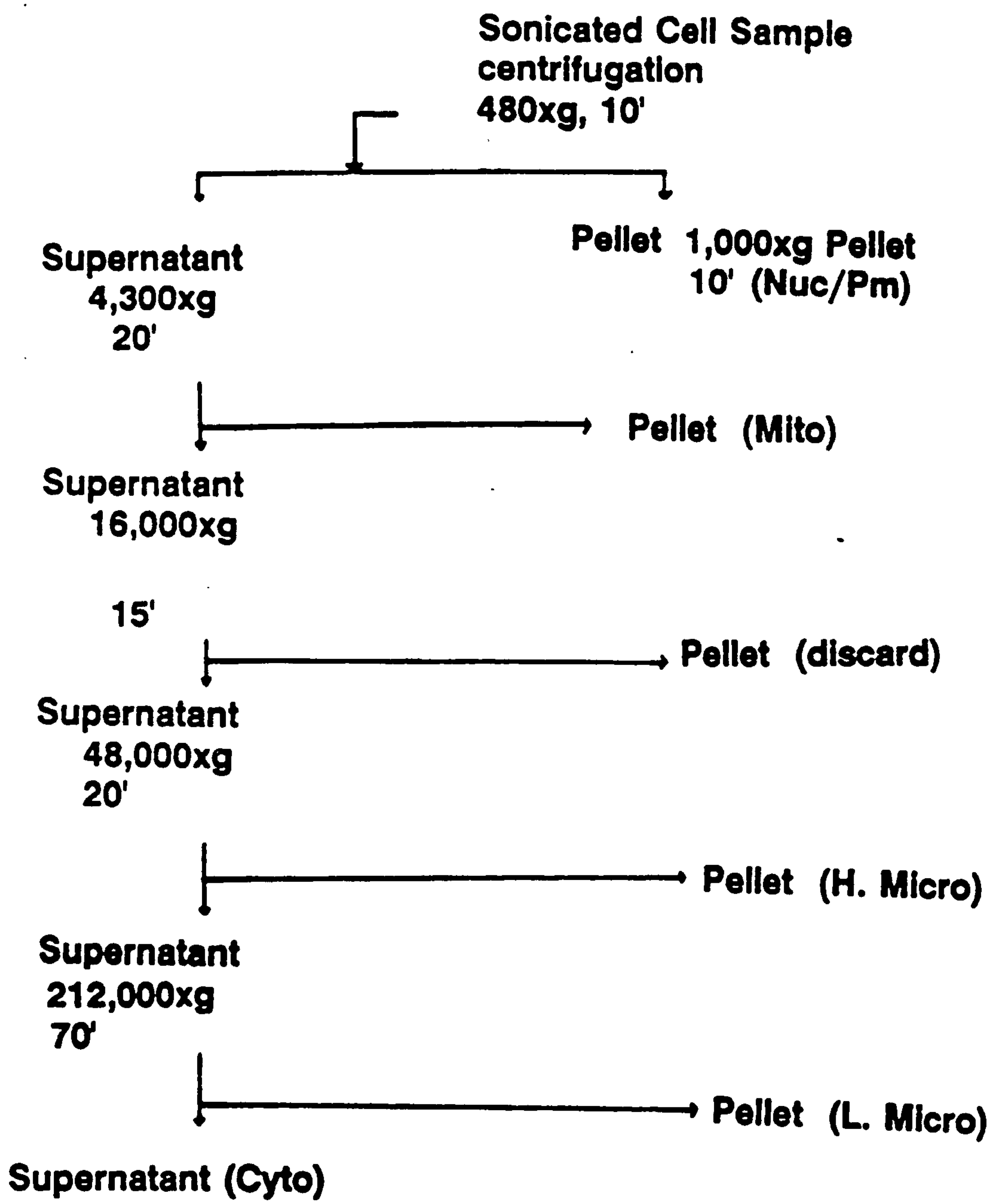

Sonicated Cell Sample
centrifugation
480xg, 10'
Supernatant
4,300xg
20'
Pellet 1,000xg Pellet
10' (Nuc/Pm)
Pellet (Mito)
Supernatant
16,000xg
15'
Pellet (discard)
Supernatant
48,000xg
20'
Pellet (H. Micro)
Supernatant
212,000xg
70'
Pellet (L. Micro)
Supernatant (Cyto)

FIGURE 7

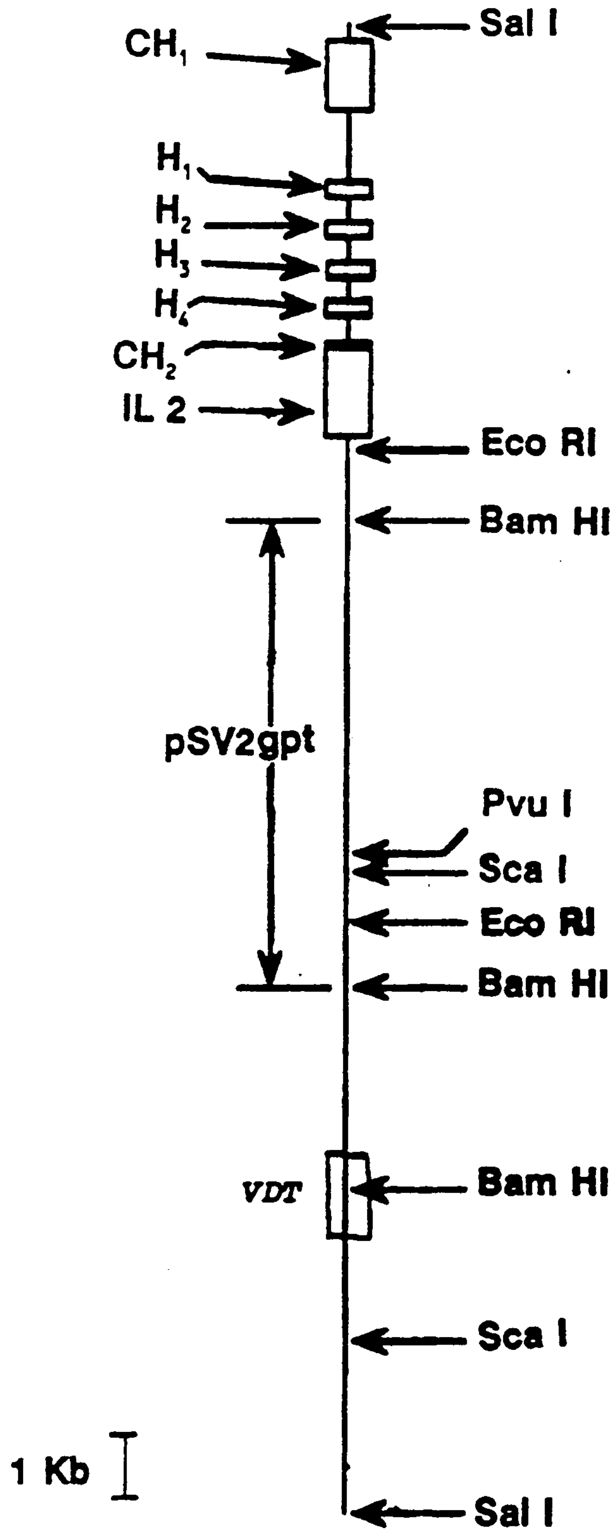
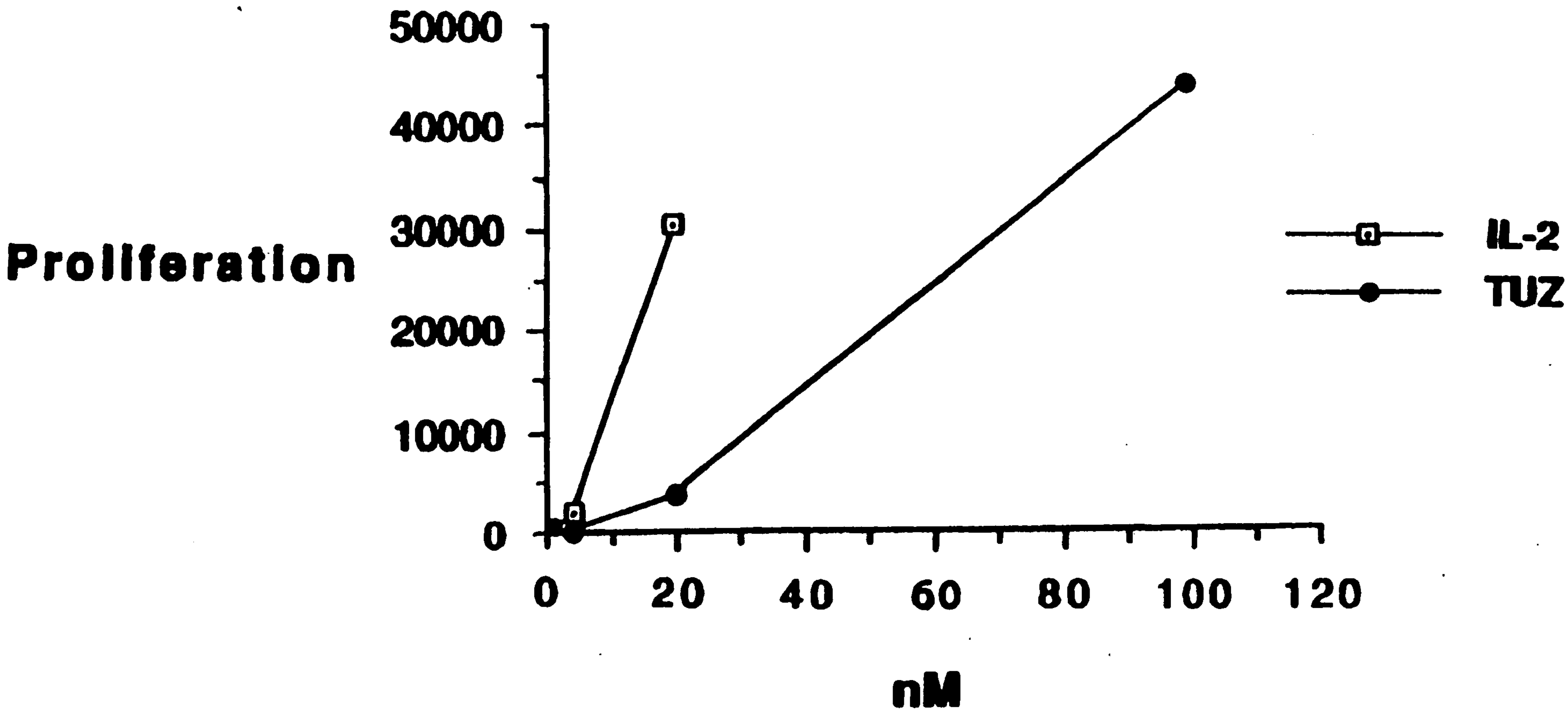

Sal I
CH1
H1
H2
H3
H4
CH2
IL 2
Eco RI
Bam HI
pSV2gpt
Pvu I
Sca I
Eco RI
Bam HI
VDT
Bam HI
Sca I
1 Kb
Sal I

FIGURE 8



Proliferation
50000
40000
30000
20000
10000
0
0
20
40
60
80
100
120
nM
IL-2
TUZ

FIGURE 9

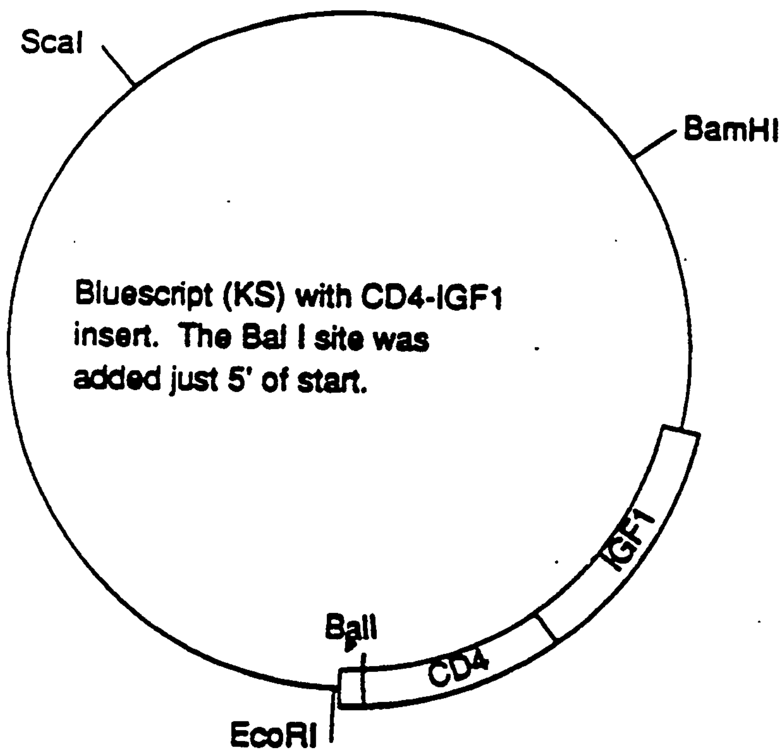

ScaI
BamHI
Bluescript (KS) with CD4-IGF1 insert. The Bal I site was added just 5' of start.
IGF1
CD4
BalI
EcoRI

FIGURE 10

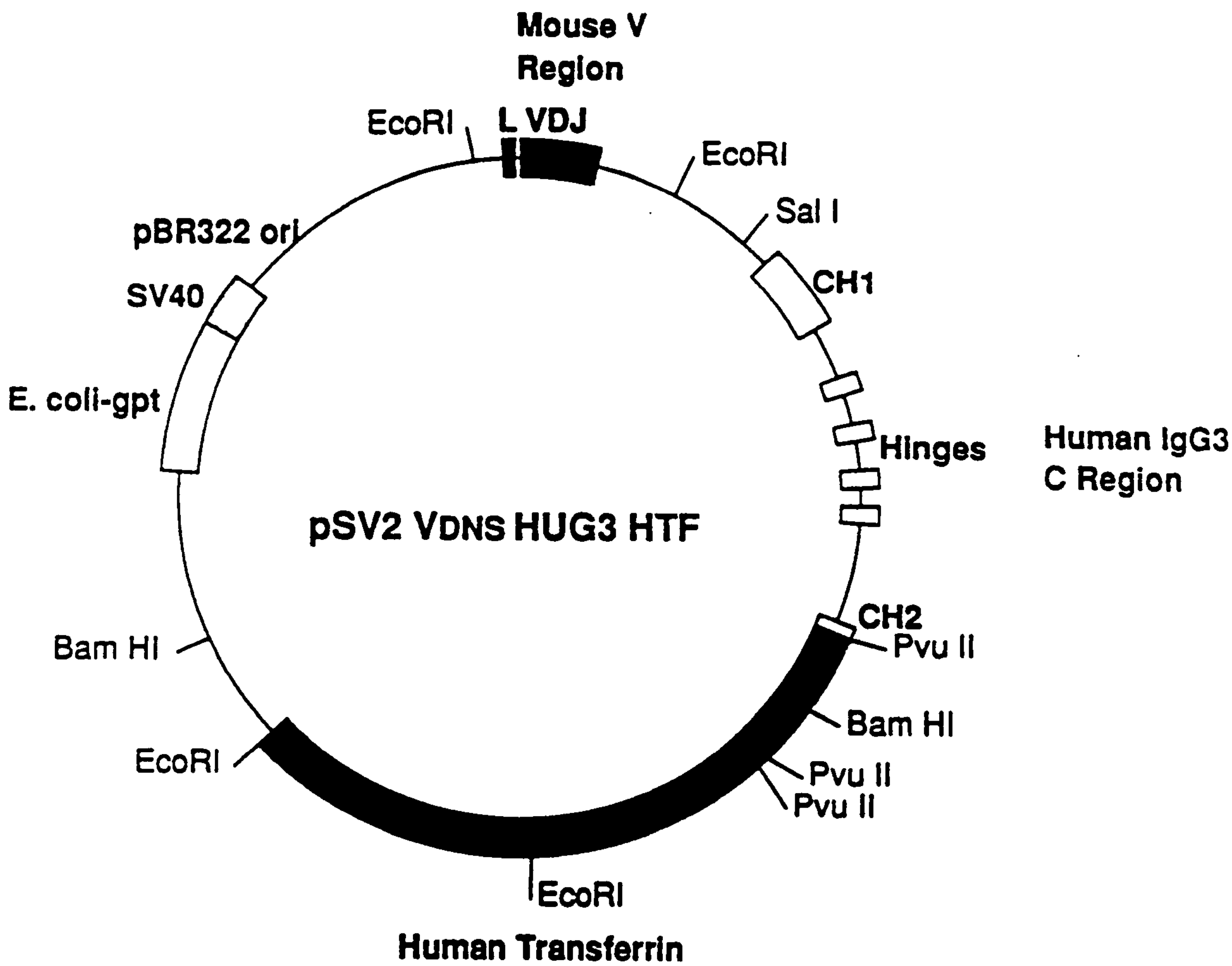
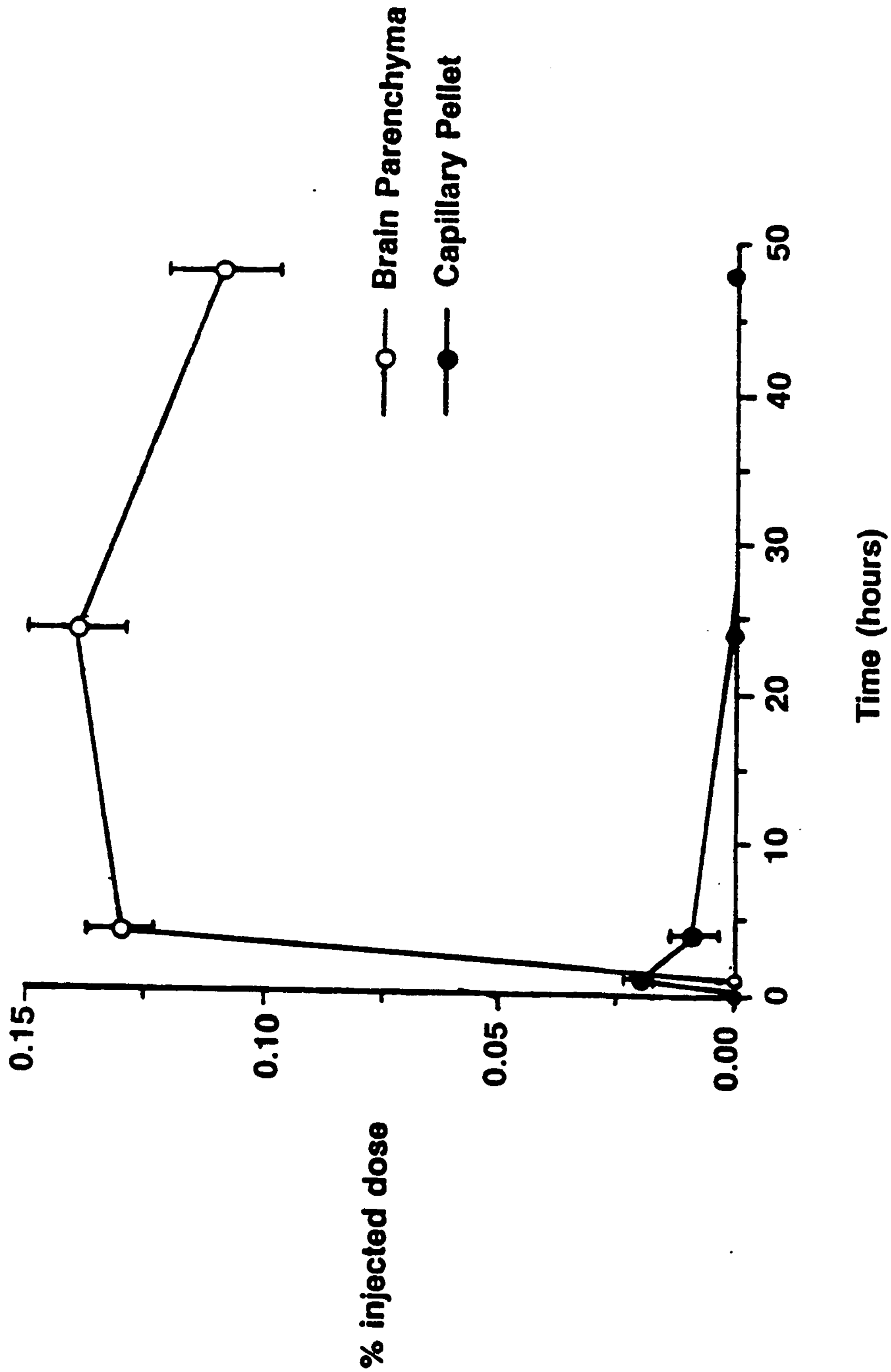

Mouse V
Region
EcoRI
L VDJ
EcoRI
Sal I
CH1
pBR322 ori
SV40
E. coli-gpt
Hinges
Human IgG3
C Region
pSV2 VDNS HUG3 HTF
CH2
Pvu II
Bam HI
Bam HI
EcoRI
Pvu II
Pvu II
EcoRI
Human Transferrin

FIGURE 11



% injected dose
0.15
0.10
0.05
0.00
0
10
20
30
40
50
Time (hours)
Brain Parenchyma
Capillary Pellet

INTERNATIONAL SEARCH REPORT

International Application No PCT/US91/01844

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC: A61K 35/14, 37/04, C07K 15/22, 13/00, C12 P21/02 C12N 5/00

US: 530/387, 405; 424/85.8; 435/70.21, 240.27

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl.	530/387, 405; 424/85.8 435/70.21, 240.27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Methodol Surr. Biochem., Anal., Vol. 15, issued 1985, Winter et al., "Anote on restructuring Enzymes and antibodies.", pages 139-140, see abstract No. 1364SOM.	1-20,27-32, 38-59,72-75, 78-85,119-122
P,X Y	Proc. Natl. Acad. Sci., Shin et al., Vol. 87, issued July 1990, "Expression and Characterization of an Antibody binding specificity joined to insulin-like growth factor 1: Potential applications for Cellular targeting," pages 5322-5326, see entire article.	1-11,27-32, 38-50,66, 72-75,85-91,98,99, 119-122 51-59,78-85, 92-97
Y	Nucleic Acid Research, Clarckson et. al., Vol. 17 No. 24, issued 1989 "Sticky feet-directed mutagenesis and its application to swapping antibody domains" pages 10163-10170, see materials and methods.	1-20,27-32, 38-59,66,72-75,78-99, 119-122

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 June 1991	10 JUL 1991
International Searching Authority	Signature of Authorized Officer
ISA/USA	Lila Feisee

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US,A, 4,816,567 (Cabilly et al.) 28 March 1989, see cols. 5-10, claims 1-7.	1-20,27-32, 38-59,66,72-75,78-99, 119-122
Y	Science, Vol. 238, issued 20 November 1987, Vitteta et al., "Redesigning Natures Poisons to Create Anti-tumor Reagents," pages 1098-1104, see entire document.	78-99

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers ____________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

see telephone practice.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
   1-20, 27-31, 38-59, 66, 72-75, 78-85, 91-99, and 119-122
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.